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Editors

Molecular Typing in Bacterial Infections

Humana Press

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Part I
General Consideration on Microorganism Typing Methods

Chapter 1
Molecular Epidemiology

Amy E. Seitz and D. Rebecca Prevots

1.1 Introduction

Epidemiology is the study of the distribution and determinants of disease in populations [1, 2]. Although historically studies have combined epidemiologic methods with molecular techniques available at the time, such as antibody titers for leptospirosis [3] or blood lipids for cardiovascular disease epidemiology [4], only in the last few decades has molecular epidemiology been identified as a unique field. Early definitions and utilization of molecular epidemiology in research appeared in the literature in the late 1970s and early 1980s [5–7]. These definitions included the combination of "sophisticated" or "advanced" molecular laboratory techniques with epidemiologic methods [5, 7]. Utilization of molecular epidemiology became more common in the literature around this time as well [6, 8, 9]. Technological advancements in molecular biology and genetics are contributing to the increasing integration of these techniques into modern epidemiologic research as molecular epidemiology.

One hallmark of molecular epidemiology has been the integration of biomarkers with epidemiologic methods for precisely defining exposure, disease, markers of disease susceptibility or intermediate endpoints between exposure and disease [10, 11], particularly in the field of chronic disease epidemiology. More recently, the incorporation of molecular tools in infectious disease epidemiology has resulted in the ability to more precisely describe pathogen genetic variation in human populations and how this variation relates to disease causation, distribution, and transmission.

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Biomarkers used to detect exposure and intermediate endpoints in cancer and chronic disease molecular epidemiology can be described as fitting into a continuum between biomarkers of exposure and biomarkers of disease as described by Perera et al. [7]. Internal dose, the first area of biomarkers on the continuum, is the amount of an infectious agent, substance, or metabolite measured in a biologic sample [7, 10–12]. Examples include cotinine levels in the blood to detect exposure to nicotine or blood lead level for lead exposure. The biologically effective dose is the amount of the internal dose that interacts with the host to cause disease [7, 10–12]. Early biologic effects and altered structure or function are usually correlated with the health effects from the exposure and can represent alterations in an aspect of the host system that has been changed due to the presence of the exposure chemical [7, 10–12]. An example in cancer molecular epidemiology is the presence of DNA adducts, sections of DNA bonded to carcinogenic chemicals.

Host biomarkers of exposure, infection, and genetic determinants of susceptibility to disease, similar to the continuum described by Perera et al. [7] for cancer and chronic disease epidemiology also exist in infectious disease molecular epidemiology. As with the examples from cancer and chronic disease biomarkers, distinct categories with these biomarkers may not exist for all points on the continuum for all disease processes. The initial exposure from pathogenic organisms able to cause infection is described by the infective dose, the number of pathogenic organisms entering the host sufficient to cause disease. An example of a biomarker to detect initial uptake of organisms could be identification of amplified DNA using polymerase chain reaction (PCR) for samples from blood or lymph system to determine the presence of an organism such as *Treponema palladium*.

Detecting a host immune response to the pathogen indicates exposure and infection. Antibody titers and antigens are commonly used to indicate an immune response and a history of exposure to an infectious agent in a population. In particular, age-specific patterns of immunoglobulin G (IgG) are used to indicate cumulative history of exposure to infectious agents, assuming that a good serologic correlate of infection exists. An example is the age-specific seroprevalence of IgG antibodies to serogroup C obtained as part of a serosurvey conducted to monitor the impact of introduction of a *Neisseria meningitidis* serogroup C vaccine in Australia [13]. For mycobacteria such as tuberculosis or nontuberculous mycobacteria, skin testing using purified protein derivatives (PPD) can indicate history of exposure in specific populations [14].

The relationship between infection and disease is complex and will depend on the virulence of the organism as well as the immune status and genetic susceptibility of the host. The host genetic makeup will include polymorphisms in alleles for genes involved in the immune response. Not included in the continuum of biomarkers of exposure and disease as described by Perera et al. are host genetic biomarkers for susceptibility. These genetic markers are measured independently from exposure and can provide information about the probability of developing disease [12]. Specifically, single nucleotide polymorphisms (SNP) from blood or buccal samples could be used to indicate susceptibility to a specific disease. Variations in the genes

of the major histocompatibility complex have been associated with susceptibility to diseases such as leprosy [15] and tuberculosis [16, 17]. Defects in the complement pathway can affect the host immune response to *N. meningitidis*, leading to invasive meningococcal disease (MD). Functional polymorphisms in the complement factor H (fH) pathway promoter lead to higher plasma concentrations of fH which have been associated with susceptibility to MD, particularly that due to serogroup C disease [18].

Finally, altered structure or function in the host can result from infection with a pathogen, leading to disease manifestations or sequelae. Toxins such as the shiga toxin from *Shigella dysenteriae* can affect the capacity of the kidney to handle water by causing damage to specific cells [19]. The presence of shiga toxin could be used as a biomarker of this stage of infection.

Using biomarkers in epidemiologic studies adds to the strength of the study design and the specificity of measured associations by reducing misclassification errors: exposure or disease can be more precisely detected [11]. Relying only on questionnaires which assess the amount or time frame of exposures allows the possibility of recall bias, especially if a long period of time has passed since the exposure or if the exposure was not memorable. Disease misclassification can occur if the symptoms are nonspecific or if the disease presents differently among patients. Using biomarkers to identify an exposure or disease in an individual can provide a quantifiable way to classify exposed and/or diseased persons. The most commonly used biomarkers in infectious disease epidemiology are antibodies, which provide a specific measure of past or present exposure and infection.

Although immunologic and biochemical methods have been used historically to differentiate pathogens, more precise methods based on advancements in genomic technology have allowed more complete delineation of transmission dynamics and pathogenesis of disease in populations. An abundance of genetic information on hosts and pathogens has contributed to these advancements [20]. Specifically, these tools provide the researcher a way to characterize and identify the relatedness of one organism to another organism, either through unique structural molecules such as surface proteins, or by identifying unique genetic patterns such as using RFLP to detect changes in DNA or RNA banding patterns based on cleavage at specific restriction sites or sequencing to detect variation in individual base pairs [21]. These methods also allow the researchers to measure changes in the host in response to the exposure or pathogen.

Genomic technology in molecular epidemiology generally falls into one of three categories: (1) DNA banding patterns, (2) DNA hybridization, and (3) DNA sequencing [22]. Techniques such as pulse field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), PCR, and other specific assessments of DNA sequences such as chromosome comparison and ribosomal DNA comparison can be used to characterize an organism by DNA "fingerprints." PCR, PFGE, and RFLP have been in use since the mid-1980s [23–27]. Multilocus sequence typing (MLST), developed in the late 1990s, characterizes organisms by identifying internal gene fragments of housekeeping loci [28]. MLST was initially developed

for use with *Neisseria meningitidis* but has been used for other organisms such as *Campylobacter* and *Staphyloccocus* [28–30]. Spoligotyping, also developed in the late 1990s [31], provides a rapid method for *Mycobacterial tuberculosis* strain differentiation. A more recent method, mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing, was developed in 2001 [32] and is a high resolution typing method for *Mycobacterium tuberculosis* based on specific genetic elements in the *M. tuberculosis* genome.

Advances in genetic sequencing, including reduced cost and processing time, have allowed these methods to become powerful tools in the field of molecular epidemiology. One example is the use of Genome Wide Association Studies (GWAS). GWAS take advantage of SNPs to use as biomarkers for genetic susceptibility to disease. GWAS commonly include a wide panel of potential alleles to detect variant alleles which could be determinants of host disease susceptibility.

Additionally, molecular epidemiology has greatly benefited from our ability to rapidly transmit, share and store information. The now common practice of sharing and storing sequences on widely accessible databases has enhanced the potential of molecular epidemiology and provided a unique approach for sharing and describing global epidemiology of pathogen strains. GenBank [33], a publically accessible database maintained by the National Center for Biotechnology Information at the National Institutes of Health, includes over 100 million sequences of various organisms and is widely used for molecular epidemiology.

With the recent advancements in genetic analysis, we are now able to differentiate between organisms at the level of the nucleotide, identify altered structure or function within the host or identify individuals who may be most susceptible to infection or disease and obtain rapid access to large databases. These advancements allow for reduced error in measurement of exposure, infection, susceptibility, and disease, or an intermediate state, and have greatly enhanced the field of molecular epidemiology and epidemiologic analysis in general.

1.2 Current Applications of Molecular Epidemiology

1.2.1 Surveillance and Food Borne Illnesses

One current application of molecular epidemiology to benefit public health is the National Molecular Subtyping Network for Foodborne Diseases Surveillance (PulseNet), a national network of public health laboratories coordinated by the Centers for Disease Control and Prevention [34]. PulseNet uses PFGE to obtain genetic fingerprints of disease causing bacteria from infected humans and suspected food items. These genetic fingerprints are maintained in a database which allows for rapid communication and information dissemination among public health laboratories. This information allows epidemiologists to quickly detect geographically or temporally distinct clusters, distinguish between concurrent but unrelated illness, and determine the source of infection [34].

1.2.2 Geographic Distribution

A primary question in the very early stages of investigating a possible outbreak is "Are cases of the disease that we are seeing in one geographic location epidemiologically related to cases in other geographic locations?" Determining the extent of geographic distribution of the epidemic clones and their genetic relatedness is an important aspect of molecular epidemiology. Furthermore, cases may present as isolated or "sporadic" cases; molecular epidemiologic methods can be key to determining whether seemingly unconnected cases can be attributed to a single source [35].

A study published in 2008 by Lomonaco et al. used molecular epidemiology to determine the relatedness of *Salmonella* cases [36]. Using serotyping, PCR, and PFGE, the study investigators were able to compare isolates of *Salmonella* from food items and human cases to determine the degree of similarity between isolates and determine if there was a common source of infection. The use of these combined epidemiologic techniques identified a possible connection through contaminated eggs and sausage between clusters of *Salmonella* cases, spread over multiple counties, which were not previously suspected to be linked [36].

Staphylococcus aureus is well known mostly for the serious public health challenges that it presents regarding its resistance to antibiotics such as methicillin [37]. The clonal structure for *S. aureus* is relatively stable and can therefore provide a strong indication of the relatedness of multiple infections [37]. Specifically, sequencing of the X-region of the *spa* gene to identify *S. aureus* clones has been shown to be more specific than phage typing and therefore especially useful for identifying sources in outbreaks [38]. A 2010 study compared sequences in a specific region of the *spa* gene from *S. aureus* for epidemiologic typing to determine geographic distribution and transmission information of methicillin resistant (MRSA) and methicillin susceptible *S. arueus* (MSSA) in Europe [39]. The study investigators were able to identify unique isolates that share common ancestry, allowing them to determine the distribution patterns of clonal clusters of MRSA and MSSA across Europe. They found increased regional clusters of MRSA as compared to MSSA, possibly indicating a stronger epidemic behavior among MRSA as compared to MSSA. They hypothesized that the geographic clustering of MRSA was due to the spread through networks of healthcare facilities rather than spread through the general population outside of healthcare facility networks [39].

Aikembayev et al. used molecular epidemiology to describe historic patterns in the geographic distribution of outbreaks and strain subtypes for *Bacillus anthracis* in Kazakhstan [40]. Using SNP typing and multilocus variable number tandem repeat analysis (MLVA), they were able to identify the dominant isolates of *B. anthracis* and provide a historical perspective by linking these to a lineage previously identified by isolates in Europe. In addition, the use of molecular epidemiology in this study allowed for the identification of novel genotypes of *B. anthracis* [40].

1.2.3 Temporal Distribution

The distribution of a disease through time can provide insight into the history of the human populations affected as well as the evolution of the organism and its change in response to selection pressures. Knowing this information, including the degree of polymorphisms or mutations, is important for understanding the stability of the organism and in determining what degree of change might be expected over a certain amount of time; these findings can in turn guide development and modification of control strategies such as vaccination or development of new antibiotics.

Feikin et al. collected serogroup information for pneumococcal isolates reported in multiple studies in the United States from 1928 to 2008 to assess temporal epidemiological trends in invasive pneumococcal infection over the time period [41]. The investigators found a decline in pneumococcal serogroups 1–3 and 5, which they hypothesized was due to the introduction of sulfa antibiotics in 1936 and the later introduction of penicillin [41].

A study from 2002, using amplified fragment length polymorphisms (AFLP) analysis to characterize the isolates, investigated the relatedness of *Bartonella bacilliformis* isolates from Peru [42]. The investigators identified isolates unique to what is typically encountered in the region. The study also identified a similar genotype among isolates collected 40 years ago and more recent isolates, indicating high stability in the *Bartonella bacilliformis* genotype [42].

1.2.4 Mode of Disease Transmission

Molecular epidemiology can provide useful tools for determining the mode of transmission of an infection, and the proportion of disease which may be attributable to a single clone. *Burkholderia cepacia* has been known to cause outbreaks of infection among individuals with cystic fibrosis, usually from common source nosocomial outbreaks. It is also known to cause nosocomial outbreaks among patients without cystic fibrosis due to a common source [43].

Holmes et al. used molecular epidemiology tools to identify a third type of *B. cepacia* outbreak involving possible person-to-person transmission between cystic-fibrosis and non-cystic-fibrosis patients in a hospital setting [43]. The study investigators used ribotype RFLP profiles and PFGE to determine the relatedness of isolated clones from the affected patients and possible environmental sources. Results from ribotype RFLP and PFGE identified a single clone of *B. cepacia* in patients with and without cystic fibrosis. They were not able to identify environmental sources that could have served as a common source for the transmission. Cystic-fibrosis patients with *B. cepacia* were isolated according to protocol to prevent transmission although compliance was incomplete. In addition, non-cystic-fibrosis patients with *B. cepacia* were not isolated. These factors, along with the lack of

evidence for a common environmental source, indicate person to person transmission as a previously unidentified mode of transmission among cystic fibrosis patients for *B. cepacia* [43].

1.2.5 Nosocomial Infections

Investigation of outbreaks in hospital and healthcare facility settings greatly benefit from the use of molecular epidemiologic tools. Determining if an increase in the number of cases of an infection is due to a single source or from multiple sources can define the actions taken to prevent additional cases from occurring.

PFGE was used to identify a single clone and source of infection in an outbreak of *Enterobacter cloacae* among newborns in a hospital in Campinas, Brazil [44]. Eleven cases of *E. cloacae* were identified among newborns in the hospital in a 16-h period during June 1995. Samples were obtained from possible environmental sources, medications and intravenous fluids given to the newborns. Patient isolates of *E. cloacae* were identified using PFGE. Isolates from ten patients in the neonatal intensive care unit were found to be indistinguishable. The isolate from an eleventh case was determined to be distinct from the other ten isolates by PFGE. It was further determined that this newborn did not receive parenteral nutrition as did the other ten cases and it was concluded that this patient's infection was not due to the same source. *E. cloacae* was isolated from multi-dose parenteral solution which was in use during the outbreak. The genomic DNA profile of the *E. cloacae* isolated from the parenteral solution was identical to that of the ten patients, indicating the parenteral solution as a single source of infection for the ten cases. A second source was not identified for the eleventh case [44].

1.2.6 Transmission Dynamics and Disease Prevention

Molecular epidemiology is critical to understanding the dynamics of disease transmission to better guide control strategies. These dynamics include the mode of transmission, the virulence of the strain causing the infections, possible host carriers or reservoirs of the pathogen, spatial patterns, and degree of drug resistance. Elucidation of these features has important implications for disease prevention and control strategies. Molecular technologies have been critical to illuminating the transmission dynamics of tuberculosis, particularly through more precise differentiation of clinical isolates. The first and most widely used molecular method has been the RFLP based on analysis of the IS6110. More recently, PCR-based methods have been used for improved discrimination among clinical isolates. Of these, the analysis of mycobacterial interspersed repetitive unit-variable number tandem repeats (MIRU-VNTR) has allowed the greatest precision in identifying the relatedness or "clustering" of isolates.

Small et al. used population-based RFLP of Mycobacterium tuberculosis in combination with traditional contact tracing to describe the transmission pattern in San Francisco [45]. Using these methods, they identified 44 unique clusters of infection each containing at least 2 individuals. A total of 191 patients had strains that were linked to one of the 44 clusters from a total of 473 patients. They also noted that conventional contact tracing was able to identify only about 10% of the individuals in the clusters which they identified using RFLP. After investigating the three largest clusters that they identified, they found that all of the index cases had low compliance with treatment. Specifically, they identified one individual who was not adequately treated and subsequently infected 29 additional individuals [45].

A recent study of multidrug resistant tuberculosis (MDR TB) in South Korea using both RFLP and spoligotyping\MIRU-VNTR, found a low level of clustering of isolates, suggesting that acquisition of drug resistance through inadequate treatment strategies, rather than transmission of resistant strains, is the primary public health concern [46]. In addition, these molecular methods were useful in identifying a cluster related to nosocomial transmission within this setting of largely primary resistance [46]. Similarly, a study in South Africa of extensively resistant *M. tuberculosis* (XDR-TB) strains among HIV infected patients using molecular methods identified a high degree of genotypic diversity among the isolates [47]. They concluded from these results that a majority of the cases of XDR TB were a result of primary acquisition of MDR TB through incomplete treatment rather than through primary transmission [47].

Molecular epidemiology can aid in vaccine development through identification of trends in vaccine and non-vaccine strains of organisms. For example, vaccines developed to prevent invasive pneumococcal disease are designed to provide protection against specific serogroups and serotypes [48]. The molecular epidemiology of pneumococcal diseases, specifically the serotypes circulating in the population, is needed to guide pneumococcal vaccine development. The pneumococcal conjugate vaccine containing seven of the most common isolates in the United States (PCV-7) was developed for use in the United States in 2000 and its usage has since expanded globally [48]. More recent versions of the pneumococcal conjugate vaccine contain additional isolates [49, 50]. Similarly, vaccines to prevent meningococcal disease (MD) from *Neisseria meningitidis* have been developed for serogroups A, C, Y, and W-135. However, more precise serologic and molecular characterization of isolates has been critical to development of vaccines. In New Zealand, an epidemic of meningococcal disease that lasted more than 14 years was associated with a single clone of NM serogroup B, the B:4:P1.7-2,4 clone which had caused 86 % of cases during 1986–2003 in New Zealand. In an unprecedented effort, an Outer Membrane Vesicle (OMV) vaccine was developed, targeted specifically against this strain. This strain was licensed, and from 2004 to 2006, three million doses were administered to one million persons aged <20 years. Routine vaccination of infants continued until June 2008 [51]. This type of vaccine development would not have been possible without the development of precise seroepidemiologic tools to characterize the strains. A molecular typing scheme has been recently proposed which incorporates both genes coding for surface antigens as well as more conserved genes based on

MLST which distinguish genetic lineages (clonal complexes) [52]. Application of this system to meningococcal isolates in England showed that 80 % of isolates belonged to a single clonal complex, ST-11, and combinations of this clonal complex with the dominant porA and fetA genes could cover approximately 80 % of the targeted population [52].

1.3 Summary

The integration of molecular tools with traditional epidemiologic methods has expanded in recent years due to innovations and advancements in the molecular and genomic techniques. Because of these advancements, determining the source of infection, modes of disease transmission, geographic distribution, and temporal relatedness can now be done with great precision. As a result of this increased utility, molecular epidemiology has now become an integral component of infectious disease epidemiologic research.

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Part II
Gastrointestinal Pathogens

Chapter 2
Enterococcus

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2.1 Introduction

The enterococci are a diverse and versatile group of bacteria with several intrinsic characteristics that allow them to survive and grow under a variety of conditions and a remarkable metabolic adaptability in order to fulfill diverse roles as commensals and as opportunistic pathogens. These microorganisms are widely distributed in nature, mainly on the mucosal surfaces of humans and animals, but they are also found in soil, water, dairy products and other foodstuffs, and on plants. Under certain circumstances, they are able to cause a variety of infections in humans and are now recognized among the major etiological agents of nosocomial infections associated with limited therapeutic options, due to their ability to acquire resistance to most of the clinically relevant antimicrobial agents [1–3].

In years past, enterococcal infections were traditionally considered to be acquired endogenously from the patient's own normal flora, and the epidemiology of enterococcal infection attracted little attention. This perspective has dramatically changed and a major interest has focused on the epidemiology of enterococcal infections, because of the increasing documentation of *Enterococcus* as a leading nosocomial pathogen. Furthermore, the emergence and dissemination of multiple antimicrobial resistance traits among enterococcal strains and the evidence supporting the concept of exogenous acquisition of enterococcal infections have generated an

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additional need for typing the isolates as a means of assisting infection control and epidemiological studies both within and among various medical institutions. Therefore, the investigation of epidemiological aspects of nosocomial outbreaks as well as the dissemination of enterococcal strains harboring antimicrobial resistance markers is of major interest, particularly in the light of the increasing occurrence of vancomycin-resistant enterococci (VRE). Ideally, besides outbreak analysis, the methods used for epidemiological investigation of enterococcal isolates must be able to track enterococcal dissemination in different environments and hosts, and the evolution of multiresistant strains.

2.2 Characteristics and Current Classification of the Genus

The genus *Enterococcus* is composed of Gram-positive cocci that occur singly, in pairs or as short chains. They are non-sporing, facultatively anaerobic, catalase-negative bacteria, with a fermentative metabolism resulting in L(+) lactic acid as the major product of glucose fermentation.

Characteristics such as growth in broth containing 6.5% NaCl and hydrolysis of esculin in the presence of bile salts (bile–esculin [BE] test) are useful to identify enterococcal strains. Other characteristics presented by most enterococci include hydrolysis of leucine-β-naphthylamide (LAP) and L-pyrrolidonyl-β-naphthylamide (PYR) [3, 4].

The enterococci were earlier considered as a major branch within the genus *Streptococcus,* distinguished by their higher resistance to chemical and physical agents and accommodating most of the serological group D streptococci. After the introduction of molecular methods for studying these microorganisms they have undergone considerable changes in taxonomy, which started with the recognition of *Enterococcus* as a separate genus [5]. *Streptococcus faecalis* and *Streptococcus faecium* were the first species to be transferred to the new genus as *Enterococcus faecalis* and *Enterococcus faecium*, respectively. The continuous use of molecular approaches has allowed major developments in the classification of the enterococci, resulting in the recognition of about 35 enterococcal species to date [3, 4, 6]. The current criteria for inclusion in the genus *Enterococcus* and for the description of new enterococcal species are based on a combination of phenotypic tests and different molecular techniques, including DNA–DNA reassociation experiments, 16S rRNA gene sequencing, and whole-cell protein profiling analysis. Partial or nearly entire sequencing of the 16S rDNA is considered a practical and powerful tool in aiding the identification of enterococcal species: it has been performed for all currently recognized species of *Enterococcus*, and sequences are available from the GenBank database (www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide).

In diagnostic laboratory settings, identification of enterococcal species is generally accomplished by using a series of conventional physiological tests (see references 3, 4, and www.cdc.gov/ncidod/biotech/strep/strep-doc/index.htm for details). Several miniaturized, manual, semiautomated, and automated identification systems are commercially available and may be an alternative for the phenotypic

identification of enterococcal species in routine diagnostic laboratories. The application of molecular techniques for the rapid identification of *Enterococcus* species has also been expanded for use in clinical microbiology laboratories. A variety of molecular procedures have been proposed for the identification of enterococcal species, and with future improvements may also become widely available for the rapid and precise detection of enterococci directly in clinical samples [4, 7].

2.3 Clinical Significance and Epidemiology

The enterococci can act as opportunistic agents of infections, particularly in elderly patients with serious underlying diseases and other immunocompromised patients who have been hospitalized for prolonged periods, treated with invasive devices and/or have received broad-spectrum antimicrobial therapy. The spectrum of infections caused by the enterococci includes urinary tract infections (UTIs), wound infections (mostly surgical, decubitus ulcers, and burn wounds), and bacteremia [2]. They are also frequently associated with endocarditis, intra-abdominal, and pelvic infections. Enterococcal infections of the respiratory tract or the central nervous system, as well as otitis, sinusitis, septic arthritis, endophthalmitis, may occur, but are rare. Although the enterococci can cause human infections in the community and in the hospital, these microorganisms began to be recognized with increasing frequency as common causes of hospital-acquired infections in the late 1970s, paralleling the increasing resistance to most currently used antimicrobial agents. As a result, enterococci have emerged as one of the leading therapeutic challenges when associated with serious or life-threatening infections. *E. faecalis* is usually the most frequent enterococcal species isolated from human clinical specimens, representing 80–90% of the isolates, followed by *E. faecium* that is found in 5–10% of enterococcal infections [2, 3]. However, the ratio of isolation of the different enterococcal species can vary according to each setting and can be affected by a number of aspects, including the increasing dissemination of outbreak-related strains such as vancomycin-resistant *E. faecium*.

The pathogenesis of enterococcal infections is still poorly understood. Several potential virulence factors have been identified, although none has been established as having a major contribution to enterococcal virulence. Nevertheless, epidemiological studies show the existence of clonal relationships among outbreak isolates and support the notion that a subset of virulent lineages are often responsible for infections of epidemic proportions [1, 8–10].

2.4 Resistance to Antimicrobial Agents

Resistance to several commonly used antimicrobial agents is a remarkable characteristic of most enterococcal species, and can either be intrinsic or acquired. The occurrence of acquired traits leading to high-level resistance to aminoglycosides

(HLR-A), and resistance to glycopeptides, especially to vancomycin, is of particular clinical significance due to the impact in the treatment of enterococcal infections.

The emergence of VRE was first documented in Western Europe and in the United States. Thereafter the isolation of VRE has been continuously reported, indicating epidemic proportions in diverse geographic locations. VRE strains have been classified according to phenotypic and genotypic features [11, 12], and by molecular methods for rapid detection and precise classification which have been developed, mostly based on PCR tests [13]. Nine types of glycopeptide resistance have already been described among enterococci. Each type is associated with different genetic elements, some of which, in turn, can be divided into subtypes. The *vanA* and *vanB* are considered the most clinically relevant genotypes and are usually associated with *E. faecium* and *E. faecalis* isolates, while the VanC resistance is an intrinsic characteristic of *E. gallinarum* (*vanC1* genotype) and *E. casseliflavus* (*vanC2*–-*vanC4* genotypes). The additional types of glycopeptide resistance, encoded by the *vanD*, *vanE*, *vanG*, and *vanL-vanN* genes seem to occur rarely among enterococci. Considering the high frequency and diversity of antimicrobial traits among enterococcal isolates, determination of the genetic profile of genes associated with resistance to a variety of antimicrobials may be used as additional valuable tool for epidemiology and typing purposes.

2.5 Typing Methods

2.5.1 Early Typing Methods

Early epidemiological investigations of enterococcal infections were based on classic phenotypic typing methods used to investigate the diversity among enterococcal isolates, including biotyping and antibiotyping, serotyping, bacteriocin typing, and bacteriophage typing (see ref. [4] for additional reading). Although these approaches have occasionally yielded useful information, they frequently fail to adequately discriminate among strains, and therefore, they are of limited value for comprehensive epidemiological studies. On the other hand, the use of phenotypic typing methods in conjunction with molecular typing approaches can contribute valuable information.

2.5.2 Molecular Typing Methods

The introduction of molecular techniques has substantially improved the ability to discriminate enterococcal isolates and has provided critical insights into the epidemiology of the enterococci. By using molecular typing approaches it was possible to demonstrate the exogenous acquisition of enterococcal strains by direct and indirect

contact among patients, breaking the traditional conception that enterococcal infections were endogenous in nature. Intrahospital transmission and interhospital spread have been extensively documented for antimicrobial resistant enterococci, especially VRE [4, 14, 15]. In addition to epidemiological investigations, some of the molecular typing techniques are now used to trace the dissemination of enterococci in different environments and hosts, phylogenetic relationship, and the evolution of multidrug-resistant strains, greatly expanding our understanding of enterococcal epidemiology, population structure, antimicrobial resistance, and virulence. Emergence and global dispersion of certain epidemic enterococcal clonal complexes has been identified [8–10, 16, 17].

Several molecular methods have been proposed to type enterococcal isolates as previously reviewed [4, 18]. The first molecular techniques developed for typing of enterococci were the analysis of plasmids profiles (including both plasmid composition and restriction endonuclease analysis of specific plasmids) and the restriction enzyme analysis (REA) of genomic DNA by conventional electrophoresis. These techniques may be helpful in some instances, but problems related to inconsistencies in plasmid yield and to difficulties in accurate interpretation of the electrophoretic profiles have been encountered with the use of these methods. Multilocus enzyme electrophoresis (MLEE), ribotyping, and the polymerase chain reaction (PCR)-based typing methods, such as the random amplified polymorphic DNA (RAPD-PCR) assay, and the repetitive element sequence (REP)-PCR have also been used to investigate the genetic relationship among enterococcal strains. These methods also have limitations, such as poor reproducibility and/or high technical complexity. DNA sequencing of PCR products and restriction fragment length polymorphism (RFLP) analysis of PCR products have been used to trace and to determine differences among specific resistance genes in enterococci, and therefore representing additional tools for typing resistant strains.

A remarkable contribution to the ability to discriminating among enterococcal strains was noted with the use of techniques involving the analysis of chromosomal DNA restriction endonuclease profiles by pulsed-field gel electrophoresis (PFGE) by either field inversion gel electrophoresis (FIGE) or, ideally, by counter-clamped homogeneous electric field electrophoresis (CHEF), which is the basis for most of the recent PFGE studies. Analysis of chromosomal DNA restriction profiles by pulsed-field gel electrophoresis (PFGE) has been extensively evaluated for epidemiological characterization of enterococcal outbreaks, showing improved strain discrimination and allowing the identification of clonal complexes that predominate among multidrug-resistant enterococci, mainly strains with HLR-A and VRE [4, 14, 19–21]. *Sma*I is the restriction enzyme more frequently used to digest enterococcal DNA, and the usefulness of other enzymes, such as *Apa*I and *Sfi*I, has also been documented [4].

PFGE is possibly the typing method most commonly used in clinical microbiology settings, and it is considered by many investigators as the gold standard for the epidemiological analysis of enterococcal outbreaks. Several protocols for performing PFGE typing of enterococcal strains have been published. However, the development

of standardized protocols for execution, interpretation and nomenclature, as a result of collaborative studies is still needed in order to allow for inter-laboratory data exchange and comparisons. On the other hand, although PFGE is quite discriminatory, epidemiological interpretation of PFGE profiles is not always clear-cut. The occurrence of genetic events can be associated with substantial changes in the PFGE profiles, leading to problems in clonality assessment [22]. Due to the possibility of such inconsistencies in DNA banding patterns of enterococci, PFGE is recommended mostly for the purpose of evaluating the genetic relatedness and tracing transmission of strains that are associated in time and location, as usefulness for long-term epidemiological studies may be limited. The use of PFGE in conjunction with at least one additional typing technique, or independent PFGE analysis using different restriction enzymes, is highly recommended to help clarify epidemiological interpretation. General principles proposed for the interpretation of molecular typing data based on fragment differences are usually applied to interpret PFGE profiles obtained for enterococcal strain. Well-characterized control strains should be evaluated along with unknown isolates. For that purpose, two reference strains, *E. faecalis* OG1RF (ATCC® 47077™) and *E. faecium* GE1 (ATCC® 51558™) have been proposed [23].

Two other robust molecular techniques have become available more recently for typing of enterococcal isolates: multilocus sequence typing (MLST) and multiple-locus variable-number tandem repeat analysis (MLVA). These techniques circumvent the difficulties in data exchange between different laboratories by generating information that is suitable for the development of Web-based databases. MLST is based on identifying alleles after sequencing of internal fragments of a number of selected housekeeping genes, resulting in a numeric allelic profile. Each profile is assigned a sequence type (ST). Internet sites with the possibility for data exchange have been developed (www.mlst.net, and www.pubMLST.org), which contain MLST protocols for *E. faecium* (see ref. [24] and http://efaecium.mlst.net/misc/info.asp) and *E. faecalis* (see ref. [25] and http://efaecalis.mlst.net/misc/info.asp). MLST schemes for these two species are based on sequence analysis of seven loci, each one corresponding to a separate set of different genes. Application of MLST has revealed the occurrence of host-specific genogroups of *E. faecium*, and allowed the recognition of a hospital-adapted *E. faecium* subpopulation (initially named as C1 lineage), that seems to predominate in several geographic areas [8, 9, 15–17]. This hospital-adapted lineage was later renamed as clonal complex-17 (CC17), and classified as an example of the so called high-risk enterococcal complexes (HiRECC). Figure 2.1 shows the eBURST diagram representing clusters of *E. faecium* (as of April 2010) available at the MLST database**.** Major clonal complexes have also been identified among *E. faecalis* isolates [14, 17, 25] by using MLST.

Two simultaneously published studies described the development of MLVA typing schemes for *E. faecalis* [26] and *E. faecium* [27]. MLVA is based on differences in variable-number of tandem repeats (VNTR) in multiple loci dispersed over the enterococcal genome. For each VNTR locus, the number of repeats is determined by PCR using primers based on the conserved flanking regions of the tandem

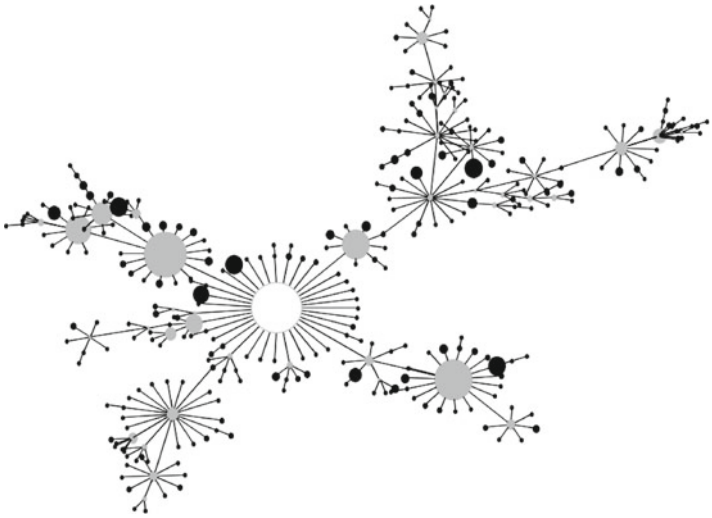

Fig. 2.1 eBURST diagram showing the clusters of *Enterococcus faecium* presently available at the MLST database (http://www.mlst.net). Each ST is represented as a *node* and the relative size of the *circles* indicates their prevalence in the database. *Lines* connect single locus variants: STs that differ in only one of the seven housekeeping genes. ST17, the presumed founder of the CC17, the major subpopulation representing hospital outbreaks and clinical infections, is represented as the *white circle*

repeats. PCR products are separated on agarose gels and the band size determines the number of repeats. These numbers together result in a MLVA profile and each profile is assigned an MLVA type (MT). The MLVA scheme for *E. faecium* is based on six VNTR loci present in noncoding regions. On the other hand, the MLVA typing scheme for *E. faecalis* is based on seven targets obtained from known genes. Figure 2.2 depicts the MLVA scheme for *E. faecium* showing typical results observed among VRE isolates from Rio de Janeiro, Brazil, belonging to a highly prevalent MT, named MT12. An Internet site has been developed (www.umcutrecht.nl/subsite/MLVA/) to serve as a database and also for the submission of MLVA profiles to assign MTs.

Comparative studies indicate that both MLST and MLVA techniques can achieve high degrees of discrimination between isolates and have comparable discriminatory power [21] that appears to be similar to that of PFGE- based typing [14, 25, 27]. In contrast to the overt advantages of being reproducible, portable, highly discriminatory and unambiguous, MLST is comparatively more expensive, and still limited to laboratories that have facilities for both PCR and sequencing, while MLVA requires PCR and basic electrophoresis facilities. Thus, MLVA may be used as an initial screening and typing method for a more rapid and less expensive alternative to MLST for clinical laboratory settings.

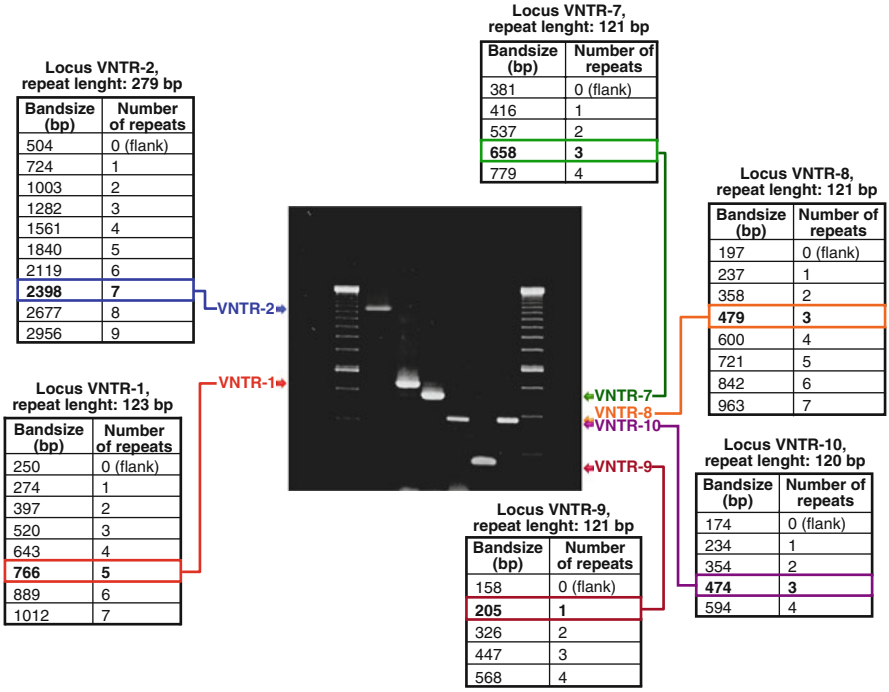

Fig. 2.2 Schematic representation of the MLVA assay for *Enterococcus faecium* isolates. Six loci are amplified by PCR, so that the size of each locus is measured and the number of repeats can be deduced. The resulting information is a code which can be submitted to the specific database (http://www.umcutrecht.nl/subsite/MLVA/). Typical results observed among VRE isolates from Rio de Janeiro, Brazil, belonging to highly prevalent MT, named MT12 (5 7 3 3 1 3), are shown in the gel

In addition to differences in complexity and costs, molecular typing methods may vary in their reproducibility and discriminatory power. Overall, there is no single definitive method to type the enterococci, so a strong match among the results of different typing techniques, particularly those based on different genomic polymorphisms, should be used as indicative of high relatedness.

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Chapter 3
Listeria monocytogenes

Todd J. Ward

3.1 *Listeria* and Listeriosis

The genus *Listeria* is a group of low G+C, Gram-positive, non-spore-forming, rod-shaped bacteria closely related to the genus *Bacillus*. The traditional taxonomy for this group recognized six species (*Listeria monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, and *L. grayi*). However, two additional species (*L. marthii* and *L. rocourtiae*) have been described recently [1, 2]. Although *L. monocytogenes*, *L. ivanovii, L. seeligeri*, and a few atypical *L. innocua* strains are hemolytic and carry copies of the *prfA* virulence gene cluster, only *L. monocytogenes* and *L. ivanovii* are generally considered to be pathogenic. *L. ivanovii* is rare and principally associated with disease in ruminants. However, *L. monocytogenes* is a facultatively pathogenic saprotroph that is widely distributed in the environment and able to cause serious invasive illness in humans and other animals.

Although invasive listeriosis is rare among healthy adults, individuals with compromised immune systems, the elderly, infants, and pregnant women have an increased risk of contracting listeriosis. The symptoms of listeriosis can be similar to those associated with influenza infections and gastroenteritis. However, *L. monocytogenes* infections can have more severe clinical presentations, including encephalitis, meningitis, septicemia, and spontaneous abortion. As a result, *L. monocytogenes* infections are associated with high hospitalization (92%) and mortality rates (20–30%), and account for over one-quarter of all deaths attributable to known foodborne pathogens [3, 4].

The overwhelming majority of listeriosis infections in humans result from the ingestion of contaminated food, and *L. monocytogenes* has a number of characteristics that create significant challenges for the food industry. *L. monocytogenes* has the

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ability to associate with biofilms, demonstrates a high resistance to ionizing radiation, can tolerate high salt conditions and low pH, and is able to grow at refrigeration temperatures. These traits allow *L. monocytogenes* to persist in food-processing environments and make *L. monocytogenes* a serious problem in ready-to-eat (RTE) meat products and cold-stored food that is eaten without significant heating [5]. Accordingly, regulatory agencies have applied a zero-tolerance policy for *L. monocytogenes* contamination in certain RTE foods, and *L. monocytogenes* has been a leading cause of food recalls due to microbial adulteration. This has significant implications for the economics of food production and processing as the estimated cost of recalls in the USA may be more than $2 billion per year [6, 7].

3.2 Identification of *L. monocytogenes*

3.2.1 Phenotypic Methods

Rapid and reliable methods for identification of pathogenic *Listeria* are of obvious importance in clinical laboratories, but also are critical to effective enforcement of food safety regulations, risk analyses, and the establishment and validation of pathogen control strategies. As *L. monocytogenes* is the only pathogenic member of the genus *Listeria* to commonly infect humans, significant attention has been focused on the development of methods for the accurate identification of this foodborne pathogen. Traditional approaches for identification of *Listeria* and differentiation of *L. monocytogenes* are based on the physiological, biochemical, or antigenic properties uniquely shared by all *Listeria* or specific to *L. monocytogenes*. Typical identification protocols, reviewed by Gasanov et al. [8], involve an enrichment step followed by growth on selective media, and take advantage of the ability of *Listeria* species to hydrolyze aesculin. Colonies that are morphologically consistent with *Listeria* are further analyzed by biochemical testing. Hemolytic activity differentiates pathogenic and nonpathogenic species, and the Christie–Atkins–Munch-Petersen (CAMP) test is commonly used to differentiate the three hemolytic species. These analyses can be combined with sugar fermentation profiles for the identification of each *Listeria* species. Commercial test strips incorporating these various biochemical tests into a single platform have been extensively validated and are included in the standard methodologies used by regulatory agencies [9, 10]. However, biochemical approaches for identification of *Listeria* species remain time-consuming and the results can be difficult to interpret [8, 11]. A variety of phenotype-based alternatives have been developed to target species-specific enzymes, antibodies, proteomic profiles, or unique light-scattering properties of bacterial colonies [8, 12, 13]. As with traditional biochemical approaches, these assays rely on the expression of specific traits and can be difficult to standardize. In addition, some of the newer phenotype-based approaches require significant investments in instrumentation that may not be widely available in public health or other microbiological laboratories.

3.2.2 DNA-Based Methods

Molecular methods for the identification of pathogenic bacteria provide a means of directly assaying variation at the DNA level as opposed to relying on the expression of particular phenotypes in order to differentiate individual species. In addition, DNA-based methods dramatically increase the amount of variation that can be exploited for species identification, and the development of these methods has increased along with the increasing availability of DNA sequence data from specific loci (16S rRNA) and from genome sequencing projects. As a first-generation approach for DNA-based identification of *Listeria* species, the hybridization of a genetic probe (single-stranded segment of DNA) to a complementary nucleic acid sequence present in the targeted species provides a simple and direct means of pathogen identification. Probe sensitivity (achieving a positive result when the target species is present in a sample) and specificity (achieving a negative result when the target species is not present in the sample) are maximized by designing the genetic probe to complement DNA sequences that are found only within the targeted species and are shared by all or most members of the targeted species. This requires prior knowledge of sequence diversity within the targeted group as well as an understanding of the distribution of sequence diversity among nontarget species that may be encountered in a sample. Gene probe approaches are simple, relatively inexpensive, and provide results that are generally easy to interpret. However, unless a nucleic acid amplification step is incorporated into the protocol, these techniques have relatively high limits of detection and are not able to detect pathogens that may be present at low levels in a sample.

Genetic variation within rRNA genes has typically been targeted in the development of genetic probes for the identification of *Listeria* species as a group, while most genetic probes designed for the specific detection of *L. monocytogenes* have targeted virulence-associated genes (reviewed by Gasanov et al. [8]). However, the Accuprobe *Listeria monocytogenes* Culture Identification Test kit (Gen-Probe, San Diego, California) is based on a chemiluminescent gene probe targeting a unique sequence in the 16S rRNA of *L. monocytogenes* [14]. This assay is among the most commonly used gene probe approaches for *L. monocytogenes* identification, and is typically employed as a means of confirming identifications based on biochemical assays, particularly when atypical isolates are encountered [9]. While the Accuprobe kit has been validated extensively, isolates of the recently described and nonpathogenic *L. marthii* produced positive reactions with the Accuprobe test that is intended to be specific for *L. monocytogenes* [1]. Phylogenetic analysis indicates that *L. marthii* is the species most closely related to *L. monocytogenes* (Fig. 3.1 and ref. [1]), and the two species apparently share highly similar sequences at the region targeted by the Accuprobe test. While *L. marthii* is unlikely to be encountered frequently in food or clinical specimens, this example highlights the importance of determining the extent and distribution of genetic variation as fully as possible prior to the development of species-specific markers.

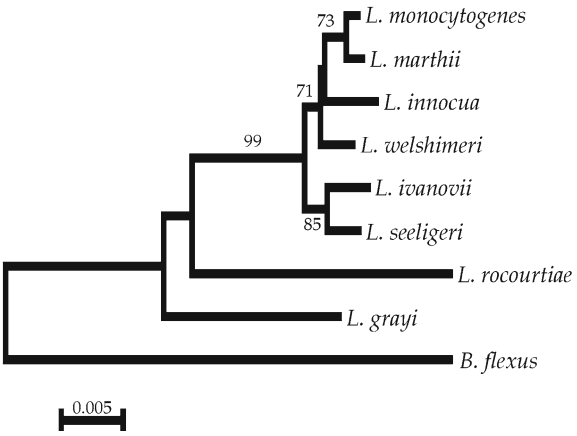

Fig. 3.1 16S rRNA gene genealogy for *Listeria*. The phylogeny was constructed by neighbor-joining analysis. The tree was rooted with sequence from *Bacillus flexus*. The frequency (percentage) with which a given branch was recovered in 1,000 bootstrap replications is shown for branches recovered in more than 70% of bootstrap replicates

Methods that utilize the polymerase chain reaction (PCR) to amplify segments of the genome containing species-specific nucleotide variation have proliferated over the previous two decades because they can offer extremely rapid and accurate results in addition to low limits of detection. The simplest PCR-based identification assays are designed such that at least one of the two PCR primers targets a species-specific region of the genome or includes species-specific nucleotide variation. These assays produce an amplification product of a specific size only when the target organism is present in the sample, and detection of the target amplicon is typically achieved via agarose gel electrophoresis. Liu et al. [11] provided a recent review of published PCR-based procedures for identification of *Listeria* species. Although many of these assays lack extensive validation and some have proven to be nonspecific, others have been extensively validated and are used by regulatory and public health agencies. For example, the USDA Food Safety and Inspection Service (USDA-FSIS), as well as several other government agencies use the BAX System PCR Assay for *L. monocytogenes* (DuPont Qualicon, Willmington, Delaware) to verify identifications that were initially based on phenotypic methods. This is an AOAC Performance Tested Method for the identification of *L. monocytogenes* based on PCR, and is reported to offer high specificity and sensitivity [15] and reportedly has a limit of detection of 10^4 cfu/ml following an enrichment of at least 24 h. It is also possible to combine sets of species-specific PCR primers into a single reaction (multiplex PCR), which enables simultaneous identification and differentiation of multiple *Listeria* species [16, 17].

The development of novel platforms for detection of PCR products provides improved sensitivity, higher throughput, and increased capacity to test for many species simultaneously. For instance, a suspension microarray approach has recently been developed for the simultaneous detection and identification of *L. monocytogenes* and seven other bacterial or viral pathogens common among meningitis patients [18]. The suspension microarray consisted of uniquely labeled microspheres coupled to oligonucleotides that complemented species-specific PCR products. Each of the microsphere complexes hybridized to its species-specific PCR product if that product was present in a sample, providing a method of sorting the various species-specific

PCR products prior to detection using a flow cytometer. This assay provided results comparable to standard phenotypic methods, but results were obtained in a single day. Similar approaches have been used to simultaneously detect a variety of common foodborne pathogens, and these technologies can be used to screen for dozens of different species simultaneously [19, 20]. Another development in PCR detection platforms, real-time PCR, eliminates end-point detection of PCR products and provides detection and quantification of PCR products as they are produced. Real-time PCR typically utilizes a species-specific probe labeled with a fluorescent reporter dye and a quencher dye. Unbound probe does not fluoresce. However, in the presence of PCR products from the target species, the probe binds to the amplicon due to the complementarity of their sequences. This uncouples the reporter dye from the quencher, permitting the fluorescence from the reported dye to be detected. Numerous real-time PCR assays have been developed for detection of *L. monocytogenes*, including a recently published assay for the detection and identification of *L. monocytogenes* directly from cerebrospinal fluid [21]. This real-time PCR assay provided results in 1.5 h and was reported to be more sensitive than culture-based methods, particularly when patients had received antibiotics [21].

Although phenotypic analyses remain the gold standard for identification of *Listeria* species, DNA-based techniques promise to improve the efficiency, accuracy, and sensitivity of *Listeria* detection and identification. With a few exceptions, the DNA-based methods for identification of *L. monocytogenes* and other species of *Listeria* require additional validation before being widely adopted, and some of the newer approaches for PCR detection require a substantial initial investment in instrumentation and training. However, real-time PCR instruments and flow cytometers are increasingly common in public health and regulatory laboratories, and significant advances in sample preparation for molecular detection are expanding the potential application of these technologies for pathogen detection in food and clinical samples which often contain components that inhibit PCR [22].

3.3 Differentiation of *L. monocytogenes* Subtypes

3.3.1 L. monocytogenes Diversity

L. monocytogenes comprises a genetically and phenotypically diverse group of strains. Not all *L. monocytogenes* strains contribute equally to human illness, and substantial differences in the ecology and virulence attributes of different *L. monocytogenes* strains have been identified (reviewed in Orsi et al. [6] and Ward et al. [23]). Therefore, the ability to differentiate individual strains or groups of strains below the species level (subtyping) is critical to detecting foodborne disease outbreaks, tracing sources of contamination, and developing risk-based inspection programs required for effective pathogen control. Improvements in molecular subtyping of *L. monocytogenes* have been driven by significant advances in our understanding of genetic diversity and its connection to phenotypic differences among strains. On the other hand, the

development of advanced subtyping technologies has enabled more detailed analyses of pathogen evolution and large-scale investigations of strain prevalence in different environments that have improved our understanding of ecological variation within *L. monocytogenes.*

Phylogenetic analyses of DNA sequences have differentiated four major evolutionary lineages within *L. monocytogenes* [6]. However, only lineages I and II are commonly isolated from food and human listeriosis patients. Lineage III is common among veterinary isolates and appears to be a host-adapted group that is poorly adapted to food-processing environments, while lineage IV is very rare and its ecological attributes remain poorly understood. In addition to lineage distinctions, classical serology has differentiated 13 serotypes within *L. monocytogenes*. However, only four major serotypes from within lineages I (4b, and 1/2b) and II (1/2a and 1/2c) account for 98% of human and food isolates. Serotyping has been one of the most common methods for *L. monocytogenes* subtyping, and while it does not provide a high level of discrimination, it has proven useful because ecotype distinctions (host-adapted strains versus environmental generalist strains) are largely congruent with major serotype distinctions. For example, serotype 4b strains are of particular concern to public health because they account for roughly 40% of sporadic listeriosis but less than 10% of food isolates. In addition, 4b strains have been implicated in the majority of foodborne outbreaks, and include three epidemic clones that were each responsible for repeated outbreaks of listeriosis in the U.S. and Europe. Serotype 4b strains also are associated with more severe clinical presentations and higher mortality rates than other serotypes. Serotypes 1/2a and 1/2b are overrepresented among food isolates, but also contribute significantly to sporadic and epidemic listeriosis, whereas serogroup 1/2c rarely causes illness and may pose a lower risk of listeriosis for humans.

Numerous genetic differences have been identified that contribute to variation in the potential of *L. monocytogenes* strains to cause disease in humans [6], but virulence-attenuating mutations in the *inlA* gene are the most common and well documented. The *inlA* gene encodes a membrane-anchored invasion protein that is critical for *L. monocytogenes* virulence. At least eighteen distinct mutations leading to premature stop codons (PMSCs) in *inlA* have been described that result in loss of the C-terminal LPXTG membrane-anchoring motif in the InlA protein [24]. Strains with these mutations display virulence attenuated phenotypes in animal models and have significantly lower invasion efficiencies in human intestinal epithelial cells [24]. In addition, strains with these mutations are significantly underrepresented among human listeriosis isolates as compared to food isolates, and serotype-specific differences in association with human illness are consistent with the frequency of *inlA* PMSC mutations within each of the major serotype groups [24, 25].

3.3.2 L. monocytogenes Subtyping Methods

Pulsed-field gel electrophoresis (PFGE) is the current gold standard for subtyping *L. monocytogenes* and most other bacterial pathogens. PFGE is performed by using one or more restriction enzymes to cut the genome into large fragments that are then

separated by gel electrophoresis in which the orientation of the electric field is alternated periodically. The resulting fragment patterns are typically analyzed and compared with the aid of image analysis software and provide a high level of strain discrimination for *L. monocytogenes*. A standardized protocol for PFGE analysis is used to subtype *L. monocytogenes* by a network of public health laboratories and regulatory agencies (PulseNet), and has proven to be useful in outbreak detection and epidemiological investigations [26, 27]. However, PFGE patterns are not always easy to interpret, and comparative analyses can become increasingly subjective as more patterns are added to PFGE databases [26]. In addition, PFGE is labor-intensive and the high discriminatory power of PFGE may be due in part to evolutionarily unstable genetic elements. As a result, PFGE types can change over very short periods of time, which may impede long-term epidemiological tracking and identification of common source outbreaks. In addition, PFGE cannot be adapted to target specific polymorphisms, such as the PMSC mutations in *inlA*, which limits the utility of PFGE data in assessing the relative public health risk associated with different *L. monocytogenes* strains. Due to these limitations, there has been significant interest in the development and validation of new DNA sequence-based methods for *L. monocytogenes* subtyping [26, 28].

Multilocus sequence typing (MLST) is a direct and robust method for molecular typing (Table 3.1; Fig. 3.2) based on variation identified in DNA sequence data derived from multiple genes (typically six or more). Unlike PFGE and other methods based on electrophoretic banding patterns, MLST data is unambiguous, highly portable, directly comparable between laboratories, and can target specific mutations or regions of interest. In addition, DNA sequence data can be used to determine the relatedness of different strains recovered during epidemiological investigations, and is ideal for use in assessing longer-term evolutionary history and population dynamics. MLST schemes have been developed for a broad range of pathogens, and several MLST approaches have been described for *L. monocytogenes* [29–31]. Although MLST schemes have typically targeted housekeeping loci, Zhang et al. [31] developed a typing scheme based on sequence data from virulence-associated genes (*prfA*, *inlB*, *inlC*, *dal*, *lisR*, and *clpP*) in an attempt to increase discriminatory power. However, the discriminatory power provided by the MLST schemes developed for *L. monocytogenes* does not appear to match that provided by PFGE [32, 33]. Discriminatory power can be increased by adding loci or sequencing whole genomes. However, this approach remains cost-prohibitive for routine use and would require significant additional training in data analysis and management. As a result, multilocus sequence data have provided significant insights into the evolution and ecological diversification of *L. monocytogenes* [6]; however, the application of MLST in epidemiological investigations and regulatory subtyping remains limited.

One of the problematic features of MLST data is that variation occurs at only a few nucleotide positions, while the vast majority of sites are invariant within the species being examined. However, the development of large comparative DNA sequence databases for diverse sets of *L. monocytogenes* strains has enabled the identification of single nucleotide polymorphisms (SNPs) that could be assayed directly. SNPs are differences at individual nucleotide positions that may be observed

Table 3.1 Comparison of the key features of PFGE and three DNA-sequence based methods for *L. monocytogenes* subtype analysis (adapted from Hyytia-Trees et al. [26])

Method	Power	Portability[a]	Objectivity of data analysis	Throughput	Analysis of strain relatedness	Ability to target specific mutations
PFGE	++++	+	+	+	+	+
MLST	+++	++++	++++	+	++++	+++
MLGT	+++	+++	++++	+++	+++	+++
MLVA	+++	+++	++++	++++	++	+

+, Low; ++, Medium; +++, High; ++++, Very High
[a]Comparability of data between laboratories

when comparing DNA sequences from different individuals, and SNP typing has become a standard approach for analyzing diversity within a wide variety of species [26]. A recently developed approach to SNP typing of *L. monocytogenes*, termed multilocus genotyping (MLGT), utilizes a suspension microarray platform (as described above) and Luminex *x*MAP fluorescent polystyrene microspheres (Luminex Corporation, Austin, Texas) to assay variation at 110 SNP sites throughout the *L. monocytogenes* genome [25, 32, 33]. MLGT provided discriminatory power that was as good as or better than MLST, but was less expensive and required fewer reactions than the MLST methods (Table 3.1; Fig 3.2). In addition, MLGT data can be used to reliably differentiate epidemiologically relevant subgroups, including major serotype groups, epidemic clones, individual outbreak clones, and strains harboring virulence-attenuating mutations in *inlA*. As with MLST, current MLGT assays do not provide the same level of discriminatory power as PFGE. However, DNA sequence-based subtyping offers a useful complement to PFGE, and the joint application of MLGT and PFGE substantially improved strain resolution among food isolates collected by USDA-FSIS [25]. For instance, five of the ten most common PFGE patterns observed in USDA-FSIS surveillance of *L. monocytogenes* were further differentiated by MLGT analysis. In addition, eight of these ten PFGE patterns included at least some strains with a virulence-attenuating mutation in *inlA* detected by MLGT. The current MLGT procedure requires a greater level of optimization and adherence to standard protocols than MLST, but SNP analyses can be performed on a variety of analytical platforms, which will undoubtedly become increasingly efficient over time [34].

Multilocus variable-number of tandem repeat analysis (MLVA) is a rapid and effective DNA sequence-based subtyping method that takes advantage of the high rate of mutation associated with short (typically fewer than 6 bp) tandem repeats of DNA sequence (Table 3.1; Fig. 3.2). The variability targeted by MLVA arises from mutations that add or delete repeat units within a tandem repeat locus, and different strains are distinguished by the fragment sizes generated by PCR amplification using primers flanking a tandem repeat. By combining data from multiple loci, MLVA can provide very high levels of discriminatory power. Sperry et al. [35] developed an MLVA method that assays variation at eight loci via two multiplex PCR reactions, with PCR products separated by capillary gel electrophoresis.

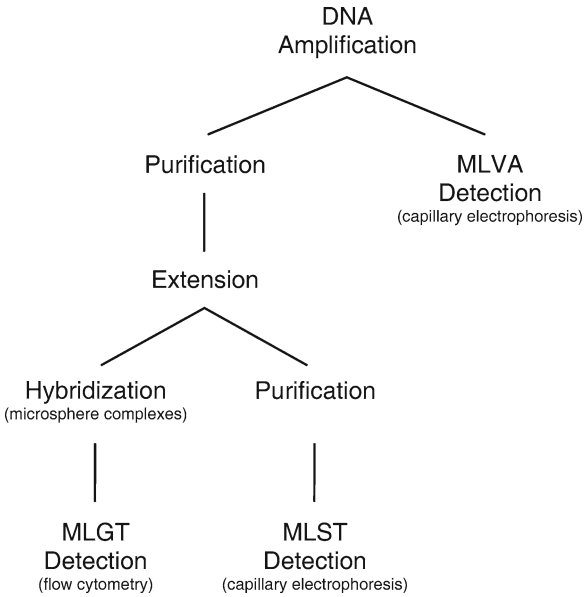

Fig. 3.2 Flow diagram depicting the major steps in DNA sequence-based subtyping of *L. monocytogenes* via MLGT, MLST, and MLVA. Note that extension, purification of extension products, and detection via capillary electrophoresis would need to be repeated for each sequencing primer used to determine the sequence of a given amplicon in an MLST analysis. The entire process would need to be repeated for each amplicon used in MLST analysis

The method provided good discriminatory power, though less than PFGE, and was able to correctly differentiate epidemiologically linked isolates from unlinked isolates. A similar MLVA system was developed specifically to differentiate the closely related serotype 4b strains [36]. These approaches are rapid, inexpensive, and potentially very powerful. MLVA can be more difficult to standardize across laboratories than is the case with MLST, and data produced by MLVA are less amenable to analyses of strain relatedness and relative risk than is the case for MLST or SNP data.

Additional subtyping approaches have been developed to differentiate specific subsets of *L. monocytogenes* strains. One of the most widely used of these methods employs multiplex PCR and agarose gel electrophoresis to differentiate the four major serogroups (4b, 1/2b, 1/2a, and 1/2c) within *L. monocytogenes* [37]. This method can't differentiate minor serotypes, but provides a very useful molecular proxy for the information provided by cumbersome serological methods. Chen and Knabel [38] also used a multiplex PCR approach to identify *Listeria*, *L. monocytogenes*, serogroups 1/2a and 4b as well as the major epidemic clones within the 4b serogroup. A targeted MLGT assay has also been developed to simultaneously differentiate isolates by lineage, major serogroup, and epidemic clone type [23]. This assay provides information to assess relative risk and is cheaper and easier to perform than MLGT assays that were developed to differentiate individual strains. Mini-sequencing reactions using the SNaPshot multiplex kit (Applied Biosystems, Foster City, California) have also been developed to provide information on the virulence potential of individual isolates by assaying all of the known mutations in *inlA* that result in premature stop codons [24, 39]. While these methods are not intended to provide the discriminatory power needed for outbreak detection and

epidemiological investigations, they provide rapid and reliable approaches for characterization of *L. monocytogenes* isolates.

Although PFGE remains the gold standard for discrimination of *L. monocytogenes* strains, further development of DNA sequence-based subtyping is likely to provide the best combination of discriminatory power, epidemiological utility, and efficiency. A variety of DNA sequence-based approaches have already been developed that could be used in conjunction with PFGE to improve strain discrimination. In addition, DNA sequence-based approaches can provide a direct means of assessing the relative public health risk posed by individual strains. These data could be used to improve future risk assessments and can be incorporated into risk-based inspection programs aimed at providing maximum protection to consumers while reducing the number of costly food recalls. Additional methodological improvements, the identification and integration of additional variation into subtyping assays, and the wider application of DNA sequence-based subtyping will almost certainly extend recent advances in our understanding of *L. monocytogenes* ecology and evolution.

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Chapter 4
Enterobacteriaceae

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4.1 Introduction

Members of the family Enterobacteriaceae are important human and animal pathogens, causing a wide range of nosocomial, zoonotic, and foodborne illnesses. Knowledge of how bacterial pathogens disseminate through the healthcare environment and food chain is important in the development of strategies to limit pathogen spread. To track these pathogens, it is necessary to identify the source and spread of infections at different stages of their transmission cycle. The ability to characterize the relatedness of strains and determine the primary sources of contamination provides valuable insights into the epidemiology and natural history of enteric pathogens. In this chapter, we explore various typing methods which can be used to differentiate bacterial pathogens. Because earlier chapters have described many of the typing techniques in detail, this chapter focuses on the strengths and weaknesses of the molecular typing methods to distinguish among members of the different species within family Enterobacteriaceae to provide insights on the best technique(s) for source tracking under different scenarios. If additional information on the basic methodology of the molecular subtyping methods is desired, there have been a number of recent high-quality reviews on the subject [1–3].

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4.2 Restriction-Based Methods

4.2.1 Plasmid Analysis

Plasmid profiling was one of the first genotypic methods used for typing enteric bacteria [4, 5]. Bacteria from the same clonal ancestry are likely to carry the same plasmids. Multiple methods have been described for the isolation of plasmid DNA from bacteria [6, 7]. These methods use selective extraction techniques for intact plasmids that minimize the isolation of chromosomal DNA. Following isolation, the plasmids are separated by gel electrophoresis, stained and viewed, and the bands compared to molecular weight standards. The number and size of the bands can be used to define plasmid profiles [8]. This profile is subsequently compared with profiles of other bacterial strains to substantiate genetic differences [2, 9]. A particular problem with plasmid profiling is the possibility of conformational changes (open circular versus closed circular supercoiled molecule) in plasmid molecules which may affect the migration properties of a particular plasmid during gel electrophoresis [10]. In some cases, two plasmids of identical molecular size, but different DNA sequences will look identical in a plasmid profile. In order to improve discrimination between bacterial strains with apparently identical plasmid profiles, an alternative genotyping method involves the digestion of the isolated plasmid DNA with restriction enzymes to generate a restriction fragment profile that is visualized following gel electrophoresis. The restriction profiles are compared to those of other isolates to distinguish among the different isolates [11]. Plasmid profiling and plasmid restriction analysis has been used for typing *Salmonella*, *Escherichia coli*, *Yersinia*, and *Shigella* [12–15]; however, many isolates lack plasmids which may limit the utility of this typing method [16].

4.2.2 Restriction Fragment Length Polymorphism Analysis

Enteric bacteria can also be compared by digesting the chromosome with a restriction enzyme and separating the DNA fragments. If a frequent cutting restriction enzyme is used, there will likely be more than 100 fragments generated that would have to be compared between the bacterial isolates, making it challenging to characterize the isolates [10]. There are two general approaches to improve the utility of restriction fragment length polymorphism (RFLP) analysis; these include the use of a rare cutting enzyme coupled with pulsed-field gel electrophoresis (PFGE) (described below) and Southern blotting. In the Southern blotting approach, the multiple DNA fragments are transferred to membranes and hybridized with a labeled probe, and the bacterial strains compared based on the restriction fragments that are homologous to the probe. One of the more common hybridization targets is the rRNA genes present in bacteria, which is further described in the section on ribotyping. A variant of traditional RFLP is PCR-RFLP, which involves PCR amplification

of specific sequences in the bacteria followed by the digestion of the PCR amplicon with a restriction enzyme to generate a DNA banding pattern [17]. The PCR-RFLP of fliC gene was used to discriminate *S. enterica* serovar Gallinarium isolates [18] and PCR-RFLP targeting the Stx-phage DNA was used to characterize *E. coli* O157:H7 isolates [19]. Others have used traditional RFLP to successfully discriminate among strains within the *Campylobacter coli* and *C. jejuni* species [17]. The RFLP method has also been used in typing *Yersinia enterocolitica* isolates using the restriction enzyme NotI to generate highly conserved RFLP patterns with fragments ranging from 15 to 400 kb [20].

4.2.3 Ribotyping

Ribotyping is a form of RFLP analysis that has been used to study the interrelationship between bacterial pathogens. Ribotyping is based on the number and location of the ribosomal RNA (rRNA) gene sequences in the bacterial genome. Differences in the sequence flanking the rRNA gene can lead to variable sized restriction fragments that are detected using Southern blotting. For *Salmonella* and *E. coli* isolates, genomic DNA has been digested with restriction enzymes such as *Pvu*II [21], *Pst*I and *Sph*I [22] and the membranes hybridized with probes that recognize the 16S and 23S rRNA gene fragments [23]. Overall, most studies have shown that ribotyping is able to genotype *E. coli* and *Salmonella* isolates either alone or in combination with other typing methods [24, 25]. However, the results generally show that the discriminatory power of ribotyping is not as high as methods such as PFGE for *E. coli* [26, 27]. Ribotyping has not been used extensively for *Yersinia*; however, when it has been used, the DNA is digested with *Sma*I and *Pst*I or *Hin*dIII and *Bgl*I [28, 29]. To date, there is also limited information available on the use of ribotyping for *Shigella* isolates [30].

4.2.4 Pulsed-Field Gel Electrophoresis

In this method, the whole bacterial chromosome is digested with a rare cutting restriction enzyme to yield a moderate number of DNA fragments which are separated using specialized electrophoresis conditions, stained, and the differences in the number and size of bands are used for comparing the genetic diversity among bacterial isolates. PFGE is considered by many as the "gold standard" for typing bacteria, particularly foodborne pathogens [3, 31]. This method is widely used under the Centers for Disease Control and Prevention's (CDC) PulseNet program to monitor the emergence and dissemination of foodborne pathogens during outbreak investigations [32]. The most frequently used restriction enzymes for *Salmonella*, *Shigella*, and *E. coli* are *Xba*I, *Bln*I, or *Spe*I [33], and for *Yersinia*, *Asc*I or *Not*I [34]. PFGE has not replaced the conventional and internationally standardized phenotypic

methods such as serotyping in *Salmonella* or Penner serotyping of *C. jejuni* [35], because PFGE tends to work better for distinguishing among isolates within a particular serovar.

Several studies have shown the usefulness of PFGE in typing *Salmonella* [9, 36]. The PFGE method has been used in conjugation with other molecular typing methods such as plasmid analysis, ribotyping, multilocus variable number of tandem repeat analysis (MLVA), PCR genotyping, RFLP, antimicrobial susceptibility testing, and multilocus sequence typing (MLST) for disease outbreak investigations involving *Salmonella*. In most cases the PFGE method was better able to discriminate between *Salmonella* isolates than other typing methods [2, 31]. There have been several reports of foodborne outbreaks associated with *E. coli* O157:H7 [37]. PFGE has been extensively used world wide for epidemiological surveillance of *E. coli* O157:H7 outbreaks [38, 39]. Similarly, PFGE has also been used in combination with other typing methods for characterizing different variants of *E. coli* isolates [40, 41]. Studies at the CDC have shown that both MLVA and PFGE methods were generally in agreement with each other in typing shiga-toxin producing *E. coli* O157 [42]. PFGE has also been used with other fingerprinting methods such as the ribotyping, plasmid analysis and PCR genotyping for subtyping *Y. enterocolitica*, *Y. ruckeri*, and *Y. pestis* [20, 33]. The PFGE method was found more suitable in epidemiological tracing of *Y. enterocolitica* isolates than other typing methods [20]. Similarly, the PFGE method has been widely for typing *Shigella* species such as *S. sonnei, S. dysenteriae*, and *S. flexneri* [43, 44].

4.3 DNA Amplification-Based Methods

4.3.1 Amplified Fragment Length Polymorphisms

Amplified fragment length polymorphisms (AFLP) analysis uses a combination of restriction digestion and PCR amplification to discriminate bacterial strains [45]. The basic procedure involves the restriction of the bacterial genome, followed by the ligation of short, complementary adaptor DNA molecules to the fragment ends that contain target DNA for specific PCR primers. The PCR primers are designed to reduce the overall number of fragments amplified and are often fluorescent-labeled, facilitating the separation of the PCR products in a DNA sequencer. The review by Vos provides a thorough overview of AFLP methodology [45]. The high level of discrimination is accomplished due to the combination of restriction analysis that produces a large number of fragments from throughout the bacterial genome and PCR amplification which serves to increase the signal of a fraction of the restriction fragments that are amplified, minimizing the amount of DNA required for analysis [45–47].

AFLP has been utilized in a number of studies for the characterization of members of the Enterobacteriaceae family. In one study, AFLP was found to be more

discriminatory than PFGE and ribotyping for distinguishing among *S. enterica* serovar Typhi isolates [48]. This method was used to discriminate among members of a specific PFGE type or ribotype. However, overall when PFGE was compared to AFLP using fluorescent primers, the distinguishing power of the two techniques corresponded well to one another [47, 49]. AFLP also appears to work well for typing *E. coli* isolates, being more able to discriminate among isolates than RAPD-PCR, PFGE, and ribotyping for isolates originating in the intensive care unit of a hospital [50]. Among *E. coli* O157 isolates, AFLP and PFGE had similar abilities to distinguish among isolates [51]. Others have used AFLP for typing *Shigella* and *Yersinia* with good success, typically being able separate isolates within particular species and serogroups [52–54]. AFLP has also been successfully used in hospital infection control schemes for the detection of disease outbreaks. Fontana et al. [55] used AFLP typing in coordination with an infection tracking software system to identify potential disease outbreaks including those caused by ESBL-producing *E. coli* and *Klebsiella* [55].

4.3.2 Random Amplified Polymorphic DNA PCR and Arbitrarily Primed PCR

Random amplified polymorphic DNA PCR (RAPD-PCR) and arbitrarily primed PCR (AP-PCR) rely on the PCR amplification of "random" or "arbitrary" sequences originating from generic PCR primers under low stringency conditions [56]. Differences in the positions of the target sequences lead to variable sized PCR products that are visualized following electrophoresis of the amplification mixtures. One of the major drawbacks of using RAPD-PCR and AP-PCR is that the reproducibility of results is sometimes questionable [57, 58]. These methods do have beneficial characteristics including taking a relatively short amount of time to genotype bacterial isolates, the requirement of only a small amount of DNA to perform the tests, and that a prior knowledge of the bacterial genome is not required for successful assay design because of the use of generic primers [59].

In general, the results of RAPD-PCR and AP-PCR have been less favorable than AFLP and PFGE for discriminating enteric bacteria. With *Salmonella*, RAPD-PCR is able to separate isolates from different serovars into their respective serovars [60]; however, the technique may be limited in discriminating within particular serovars [61]. Other researchers have better success using RAPD-PCR, being able to separate both closely and more distantly related strains of *S. enterica* serovar Enteritidis into their respective phage typing groups [62]. For *E. coli* typing, the results may be more discriminatory; in a number of instances RAPD-PCR was able to separate isolates within particular serovars [63]. However, even with the increased discrimination compared to *Salmonella* typing results, RAPD-PCR appears to be less discriminatory than PFGE for *E. coli* [64]. In *Yersinia*, RAPD-PCR also appears to be able to discriminate between isolates at the serotype level and in some cases within

particular serotypes [65]. Overall, the methods are likely less discriminatory and reproducible than PFGE and AFLP for *Yersinia* typing.

4.3.3 Repetitive Element PCR

Many members of the family Enterobacteriaceae contain sets of repetitive genetic elements in their genomes. PCR primers can be designed to amplify the regions flanking these repetitive elements. If two elements are in close enough proximity to one another the PCR reaction will proceed, and amplification products will be formed that can be separated by electrophoresis [66]. There are a number of different repetitive element PCR (Rep-PCR) methods that have been developed for bacterial typing, including the enterobacterial repetitive intergenic consensus PCR (ERIC-PCR). ERIC sequences are highly conserved 126-base pair sequences found a number of enteric bacteria [66]. Another type of Rep-PCR includes repetitive extragenic palindromic PCR (REP-PCR) [67, 68]. These REP elements are short, approximately 38 base pair DNA sequences that contain highly conserved regions of palindromic sequences that are used as targets for PCR primers [66]. Another Rep-PCR system is based on targeting BOX elements that are present in bacterial cells. For *E. coli*, the BOX A1R element has been used successfully to differentiate among non-clonal strains [69, 70].

Overall, the methods have had mixed results in their ability to distinguish between members of the species of enteric bacteria. Rep-PCR has slightly less discriminatory powers when compared to PFGE, but higher discrimination than plasmid profiling, biochemical characterization, and ribotyping [3]. In one study, there was no correlation between the phage typing and Rep-PCR in discriminating *S. enterica* serovar Typhimurium using ERIC primers [71]. The use of IS*200* primers has been a common tool for Rep-PCR of a number of *Salmonella* serovars; with serovars 4, 12:b:- and Typhimurium, Rep-PCR gave a similar degree of discrimination as PFGE [72, 73]. In contrast, PFGE proved more discriminatory than IS*200* Rep-PCR for typing serovar Heidelberg isolates [74]. The BOX A1R methodology has proven to be an effective typing tool for *E. coli* microbial source tracking [69, 75]. ERIC-PCR appears to work well to discriminate among *Y. enterocolitica* isolates; however, the results were somewhat less discriminatory that PFGE [76].

4.3.4 Variable Number of Tandem Repeat Analysis

The development variable number of tandem repeat (VNTR) analysis as molecular typing tool was facilitated by the completion of multiple microbial genome sequencing projects. One of the discoveries from this work was the fact that many bacterial genomes contain regions with repeated DNA motifs and repetitive sequences ranging from a few bases to over 100 base pairs [77, 78]. The number of copies of these

repeat motifs can be highly variable, even among strains of the same species, which allows for assays to be developed utilizing the number of tandem repeats to distinguish among non-clonal isolates [78, 79]. Strategies for using this multiple locus VNTR analysis (MLVA) have been developed for genotyping multiple members of the family Enterobacteriaceae [78, 80, 81].

For *E. coli*, many of the methods were initially developed to type serovar O157:H7 isolates [82–84]. In general the MLVA methods tend to be on par or slightly more discriminatory than PFGE for typing *E. coli* O157 isolates. Additionally, MLVA protocols have been developed for *Salmonella, Shigella*, and *Yersinia*. For *Salmonella* typing, there appears to be some serotype variability in the overall discriminatory ability [85]. With serovar Typhimurium, MLVA was more discriminatory than PFGE for typing human isolates, and the results were easier to interpret in lesser amount of time that PFGE [86]. Likewise, MLVA offered improved discrimination over PFGE for typing *Shigella* [87], and the results were comparable to the multilocus sequence typing method [87]. MLVA has also shown to be an effective tool for genotyping members of the genus *Yersinia* and *Y. pestis* in particular [88, 89]. Much of the work on *Y. pestis* has been driven by the need to develop a rapid tool to characterize this potential bioterror agent [90].

4.4 DNA Sequencing-Based Methods

4.4.1 Multilocus Sequence Typing

The increasing availability of DNA sequencing technologies has led to the development of additional new genotyping methodologies, including multilocus sequence typing (MLST). The foundation of MLST is that different strains of bacteria will have variability in the sequence of specific genes, due to mutation or recombination events, that can be utilized to determine the genetic-relatedness of bacteria. With MLST, multiple conserved genes are sequenced and analyzed to identify nucleotide base changes [91, 92]. Isolates are assigned to specific sequence types based on nucleotide polymorphism at the different loci and the sequence types are subsequently used to compare the relatedness of the isolates being studied. MLST has been used to successfully characterize a number of different members of Enterobacteriaceae. For *E. coli*, MLST has been used to discriminate uropathogenic *E. coli* (UPEC) isolates and was compared to ERIC-PCR, serogrouping and PFGE [93]. Overall, the discriminatory power of MLST was higher than that of ERIC-PCR but lower than PFGE for UPEC. When MLST, using four virulence genes, was compared to PFGE and Rep-PCR to distinguish among *E. coli* O157:H7 isolates, MLST provided the least discrimination, which may be due to the number and type of genes sequenced [41].

There have been a number of studies that have utilized MLST to determine the relatedness of *Salmonella* serovars. One study found that PFGE was much more

efficient at separating serovar Typhimurium isolates than was MLST alone when four housekeeping genes were used [94]. This result contrasts the results of other studies that found MLST was better able to discern between strains of serovar Typhimurium than PFGE [2, 95]. The differences in results among the studies may be due to the selection of genes and the number of loci that were sequenced. MLST has also been used to characterize *Shigella flexneri* [96] and assist in determining the genetic structure of species in the genus *Yersinia* [97].

4.4.2 Single Nucleotide Polymorphism Analysis

Another set of methods that utilizes the detection of polymorphic DNA sequences is SNP analysis. The single nucleotide polymorphism (SNP) analysis takes advantage of nucleotide mutations at specific loci in the bacterial genome to differentiate strains. Currently there have been few studies that have used SNP analysis to genotype *Salmonella* isolates, and those that have been done have focused primarily on analysis of genes associated with quinolone resistance [98, 99] and with flagellar antigens [100]. In a study examining *E. coli* O157:H7, a total of 906 SNPs in 523 chromosomal genes were identified [101], which likely could be used as genetic markers for high-throughput methods for genotyping *E. coli* O157:H7. As more informative SNP loci are identified in Enterobacteriaceae, SNP analysis may play a more important role in distinguishing among unrelated strains.

4.5 Conclusions

This chapter reviews the most commonly applied methods for molecular typing of pathogens in the Enterobacteriaceae family. Each of these methods has their own sets of strengths and weaknesses that make choosing the proper technique important. The current "gold standard" remains PFGE, in part because many laboratories are not yet equipped to carry out some of the more novel methodologies such as AFLP, MLVA, and SNP analysis that have shown strong promise. As these methodologies evolve and become more widely available and accepted, they may eventually replace PFGE as the dominant methods because of their ability to return results in a more rapid fashion. At present, there are multiple techniques that can be used to genotype enteric pathogens that should help to limit their spread in the healthcare setting or through the food supply. The choice of which molecular typing method to use will be dependent on the epidemiological questions being asked and the genotyping resources available to an investigator. For example, if a rapid turnaround time is needed in a disease outbreak in a confined area, such as a hospital ward, a PCR-based method may work well to link related isolates. However, if an outbreak encompasses a wide geographical area over a longer period of time, as occurs in a multistate foodborne

disease outbreak, a method such as PFGE may be more appropriate due the ability to allow comparison of typing results from multiple laboratories.

Another point to consider in evaluating the different genotyping methods is that many target different areas of the bacterial genome; thus, a phylogenetically significant difference detected by one method may not be detected by another. A single base pair change identified in SNP or MLST analysis will likely not alter a ribotype or PFGE profile for a bacterial strain unless the mutation happens to be in a restriction enzyme site. Thus, a series of methods may be needed to distinguish among truly non-clonal isolates. This multi-method typing scheme could involve initially separating isolates into phenotypic groups, such as serotypes, and then using PFGE to genotype isolates. If multiple isolates remain indistinguishable following PFGE, then MLST or AFLP could be used to further attempt to separate isolates to gain a better appreciation of the genetic diversity of the population of isolates being examined. Because of the unique characteristics of the individual genotyping methods, it is likely that the various methods will remain important tools to conduct molecular epidemiological investigations that will allow for the development of improved intervention strategies to limit the spread of enteric pathogens in the healthcare setting or through the food supply. In the future, molecular typing method development will likely attempt to improve the reproducibility and discriminatory ability of methods which return a typing result in a fraction of the time than it currently takes with conventional methods.

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Chapter 5
Molecular Typing of *Vibrio cholerae*: Imprints in the Epidemiology of Cholera

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5.1 Introduction

Cholera caused by toxigenic *Vibrio cholerae* is a major public health problem in many developing countries, where outbreaks and sporadic infections occur at regular intervals. WHO has registered 190,130 cases including 5,143 deaths with case-fatality rate of 2.7% in 2008 [1].The disease is characterized by profuse watery diarrhea that rapidly leads to dehydration, and death occurs in 50–70% of untreated patients. For more than a century, cholera remains one of the great epidemic diseases of the tropical world. Cholera has spread from Asia where it is endemic to many parts of the world in the form of seven pandemics during the past 185 years [2]. *V. cholerae* serogroup O1, biotype El Tor, has spread from Asia to cause pandemic disease in Africa and South America during the past 48 years. Until 1992, serogroup O1 was considered as the devastating cholera causative agent. A new serogroup, O139, appeared in south Asia in 1992, and changed the whole perception regarding cholera as this was the first non-O1 serogroup related with epidemic cholera. When this serogroup first appeared, it was thought that the next pandemic strain of cholera had emerged, but over the past few years the prevalence of the O139 serogroup has rapidly declined. Expansion of the seventh pandemic was accompanied by increased genetic variation among strains of *V. cholerae* O1 and O139, but the relationship of these genetic changes in relation to virulence and in epidemiology of cholera is not clearly understood.

Advances in molecular genetics have facilitated the development of refined molecular typing techniques which in turn have assisted in studying the genetic diversity of many bacterial populations. Genotyping studies related to the epidemiology of the disease otherwise called molecular epidemiology have revealed clonal

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diversity among *V. cholerae* strains and emergence of new epidemic clones over the years. Clonal information has the potential to provide information on subtypes of the organism and their source and/or origin of infection, and to recognize particularly virulent strains of the organism and monitor vaccination programs. The increasing application of procedures employing several molecular tools has provided new means of discriminating *V. cholerae*. Such studies provide a wealth of information to assist the epidemiologist in tracing and tracking the spread of epidemics and provide new insights into the evolution and origin of newer variants of *V. cholerae*. The complete genome sequence of seventh pandemic El Tor O1 strain N16961 has provided an important source to begin addressing many questions about the evolution of *V. cholerae* as a human pathogen as well as environmental organism.

Bacterial typing techniques are not always comparable as each method related to the specific research question and have its own merit. Our intention in this chapter is not to focus on the methodology of each molecular technique but to review the impact of their epidemiological applications and inference as evidenced from several studies.

5.2 Background Information on *V. cholerae*

V. cholerae was first described by Fillipo Pacini in 1854 from cholera victims followed by its rediscovery in 1883 by Robert Koch in Egypt [3]. *V. cholerae* belonging to the serogroups O1 and O139 (synonym "Bengal") are associated with epidemic and pandemics of cholera and the remaining serogroups (previously referred to as "non-agglutinable or NAG vibrios) were either nonpathogenic or causes sporadic infection. The O1 serogroup is subdivided into serotypes, i.e., Ogawa and Inaba and two biotypes, classical and El Tor. Both the serotypes are found in classical and El Tor biotypes. The first six cholera pandemics were caused by the classical biotype and the seventh pandemic has been of the El Tor biotype. Hemolysis of sheep erythrocytes, bacteriophage susceptibility with specific classical and El Tor phages, Voges-Proskauer reaction, polymyxin B susceptibility, and hemagglutination of chicken erythrocytes are used for biotyping. Though new phage typing schemes for differentiating between strains of *V. cholerae* O1 and O139 serogroups were established [4, 5], its use is limited to reference laboratories.

5.3 Polymerase Chain Reaction Based Typing

Polymerase chain reaction (PCR) has generally been used for the rapid detection of infectious agents in clinical samples or bacterial growth and later adapted as one of the molecular typing technique. PCR typing has an advantage in that the DNA can be amplified (100 to >35 kb) even if the template DNA is in minute quantity. Several PCR methods are currently available for typing of *V. cholerae*.

5.3.1 Random Amplification of Polymorphic DNA Profiles

The simplicity and discriminative capacity of this technique makes it useful for detecting genetic diversity among microorganisms from a defined group or for outbreak investigation. During 1993–94, RAPD-PCR with O139 serogroup indicated that the Asian strains were similar [6]. As shown in this study, random amplification of polymorphic DNA profiles (RAPD) patterns of the O139 strains resembled to those of E1 Tor strains rather than classical strain. Different RAPD profiles were obtained with *V. cholerae* strains from Malaysia and there was no correlation with the source of isolation [7]. A collection of *V. cholerae* strains from Brazil showed no correlation with serotype, biotype or source of the isolates [8]. However, with Iranian strains of *V. cholerae* O1, RAPD was able to discriminate into six distinct groups [9]. *V. cholerae* O1 from a 2006 outbreak in Accra, Ghana had identical polymorphic DNA profiles suggesting some genetic dissimilarity was present among the strains [10]. Generally, the discriminatory power of RAPD-PCR is low and the method is susceptible to technical variation.

5.3.2 Other PCR Based Typing Methods

The evolutionary relationships and molecular diversity of *V. cholerae* O1, O139 and non-O1, non-O139 strains were studied using amplified fragment length polymorphism (AFLP) technique. In this, two sets of restriction enzyme–primer combinations were tested for fingerprinting. Amplification of *Hind*III and *Taq*I digested genomic DNA grouped environmental isolates of O1 and non-O1 strains and was unable to distinguish between O1 and O139 clinical strains. The AFLP analyses of restriction enzyme *Apa*I and *Taq*I-digested genomic DNA separated O1 from O139 strains [11]. This study supported that a single clone of pathogenic *V. cholerae* has caused several cholera outbreaks in Asia, Africa, and Latin America during the seventh pandemic. Interestingly, some of the cholera cases were associated with environmental O1 or non-O1 strains. Castañeda et al. [12] reported a novel typing method based on *V. cholerae* repeat sequences (VCR) using specific primers. The VCR-PCR of *V. cholerae* O1 supported the hypothesis that a clone with epidemic behavior was responsible for the epidemic in Latin America.

Rivera et al. [13] have shown the usefulness of enterobacterial repetitive intergenic consensus sequence (ERIC) PCR to differentiate *V. cholerae* from a cholera epidemic in Brazil from Peruvian, Mexican and Indian epidemic strains. Majority of the toxigenic *V. cholerae* O1and O139 strains exhibited the same fingerprint (FP), whereas the non-toxigenic *V. cholerae* O1 and the non-O1 strains belonged to different FP groups. Population diversity of toxigenic *V. cholerae* was analyzed with reference to strain relatedness and their ecological interactions in the human and the aquatic habitats using ERIC-PCR [14]. In this analysis, *V. cholerae* population structure supported the hypothesis that spatial and temporal fluctuations in the aquatic environment can cause shifts in the dynamics of cholera [14]. It has been

demonstrated that typing of *V. cholerae* O1 by ERIC-PCR fingerprinting correlated well with ribotyping and was sometimes more discriminating [15]. This PCR assay provides a rapid and simple means of typing strains for epidemiological studies.

5.4 Mobile Genetic Elements

It was hypothesized that the composition of mobile genetic elements (MGEs) in *V. cholerae* strains would be useful as a phylogenetic typing system as it is conserved among *V. cholerae* O1 strains [16]. Three types of MGEs usually account for resistance to antibiotics in *V. cholerae*: (1) plasmids, which for most are large and self-transmissible by conjugation, (2) integrons, which are chromosomal- or plasmid-borne gene capture and expression systems, and (3) integrating conjugative elements (ICEs) which are chromosomal self-transmissible mobile genetic elements. In many of the epidemiological investigations, MGEs are used as typing system, especially with increase of antimicrobial resistance among *V. cholerae*.

5.4.1 Plasmids

Generally, *V. cholerae* strains are devoid of plasmids. Studies conducted for the characterization of plasmids in *V. cholerae* are mostly related to their antimicrobial resistance rather than molecular fingerprinting. Plasmid profile analysis showed the presence of 1.3–4.6 MDa plasmids in non-O1 and O1 strains from Malaysia [7]. Conjugative plasmid IncC responsible for multidrug resistance was identified in *V. cholerae* O1 strains responsible for the large cholera outbreaks in Somalia during 1998–1999 [17].

5.4.2 Insertion Sequences (IS Elements)

A 628 bp insertion sequence element, IS*1004*, is present in one to eight copies in most of the *V. cholerae* strains [18]. IS*1004*-generated fingerprints discriminated classical and El Tor but not the non-O1 strains which are heterogeneous and unrelated to those of the epidemic *V. cholerae* O1. However, with *V. cholerae* serogroup O37 that was responsible for a large diarrhea outbreak in Sudan, the IS*1004* typing showed that these strains were closely related to classical O1 strains [18]. *V. cholerae* O139 has emerged from the pandemic O1 biotype El Tor through the replacement of a 22-kbp DNA region by a 40-kbp O139-specific DNA fragment. This O139-specific DNA fragment contains an insertion sequence designated IS*1358O139*. Apart from O1 and O139 serogroups, presence of this IS sequence in multiple copies was

detected in serogroups O2, O22, and O155 but not in other non-O1 serogroups [19]. The nucleotide sequences of IS*1358* in serogroups O22 and O155 are almost identical to that of O1 and O139. The significance of IS elements found in toxigenic strains of *V. cholerae* and their non-toxigenic counterparts is not fully known.

5.4.3 Integrons and ICEs

V. cholerae contains a genetic system called the integron that can integrate and excise DNA elements by site-specific recombination. Evolution of integron arrays can proceed by rearrangements and deletions/insertions of large portions of MGEs in addition to the insertion or excision of single MGE. The extent of resistance patterns and associated MGEs in epidemic *V. cholerae* O1 El Tor was investigated in Eastern Africa with strains isolated from late 1990s [20]. This study has shown the spread of SXT-related ICEs among *V. cholerae* O1 encoding resistance to chloramphenicol, streptomycin, sulfamethoxazole, and trimethoprim in African isolates. Phenotypically, the reemerged *V. cholerae* O139 in 1995 were susceptible to co-trimoxazole compared to those that appeared in late 1992 [21]. In these reemerged strains from India and Bangladesh, a 3.6-kb region of the SXT element was deleted leading them to become susceptible to co-trimoxazole [22].

5.4.4 V. cholerae Pathogenicity Island and Vibrio Seventh Pandemic Islands

Pathogenicity Islands (PIs) are present in virulent strains comprising very large genomic regions (10–200 Kb). The G+C content of the PIs often differs from the rest of the bacterial genome, the presence of repeat at their ends and the presence of integrase determinants and other mobility loci support for the generation of PIs by horizontal gene transfer. Such changes are stable and persist for long time in the bacterial genome and hence used as a marker in bacterial typing.

V. cholerae pathogenicity islands (VPIs) can be detected in epidemic and pandemic strains of *V. cholerae* but are generally absent among nonpathogenic strains [23]. The VPI contains ToxR-regulated genes (*aldA* and *tagA*) and a part of PAI that contains a regulator of virulence genes (ToxT) and a gene cluster encoding an essential colonization factor and the toxin-coregulated pilus: TCP. Comparative sequence analysis with different strains of *V. cholerae* O1 showed polymorphism in the VPI region. There were differences in several proteins as a result of frame shift mutations [24]. Variations in the VPI region provide preliminary evidence to explain the differences in potential virulent strains appeared between epidemics. The VPI typing allowed elucidation of differences in the genetic organization between pre-pandemic and pandemic strains. Osin et al. [25] demonstrated that the genome of pre-seventh pandemic strains of *V. cholerae* O1 isolated during 1910 was devoid of CTX and

RS1prophages, Vibrio pathogenicity islands (VPI-1 and VPI-2), and Vibrio seventh pandemic islands (VSP-1 and VSP-2) that contain key virulence genes. Acquisition of VPI and CTX in *V. cholerae* was shown in cholera outbreak associated strains that were isolated during 1937. The seventh pandemic strains acquired two additional blocks of genes VSP-1 and VSP-2, which were absent in classical strains [26]. Most *V. cholerae* O1 and O139 strains carries the VSP islands (VSP-1 and VSP-2), whereas the non-O1, non-O139 strains carried several VSP island genes, but not the entire VSP island [27]. Absence of VSP islands in the Australian environmental *V. cholerae* O1 strains indicates their pre-seventh pandemic ancestry [28, 29].

In some non-O1 and non-O139 strains, the left end of VPI exhibited extensive DNA rearrangements [30]. This information suggest that potentially pathogenic, non-epidemic, non-O1 and non-O139 strains are likely evolved by sequential horizontal acquisition of the VPI and CTXΦ independently rather than by exchange of O-antigen biosynthesis regions in an existing epidemic strains.

5.4.5 CTX Prophages

Analysis of variations in the cholera toxin (CT) encoding gene (*ctxAB*) and its flanking regions serve one of the essential molecular tools for typing toxigenic *V. cholerae*. The gene *ctxAB* reside in the genome of a lysogenic filamentous phage called CTXΦ [31]. The receptor for CTXΦ is the major colonization factor, TCP. There is evidence that the island can minimally excise and therefore presumably integrate using a phage-like integrase and attachment site. The CTXΦ genome is composed of several open reading frames, located on a 4.5 Kb "core region" of the CTX element, which is essential for the morphogenesis of CTXΦ particles. Adjacent to the core is the RS2 region encoding ORFs *rstR*, *rstA2*, and *rstB2*. These genes encode products required for the integration, replication, and regulatory functions of CTXΦ. The *rstR* region is classified into $rstR^{Class}$, $rstR^{ET}$, and $rstR^{calc}$, respectively, for classical, El Tor and O139 alleles [32]. Based on the structure, organization, and location of the CTX prophages, clonal diversity was identified using restriction fragment length polymorphism (RFLP). Clonal nature of the US Gulf Coast *V. cholerae* O1 was identified with 6 and 7 kb *Hin*dIII restriction fragments that contained *ctx* gene and this pattern was not found in strains from other countries [33, 34]. *ctx* RFLP analysis was made with several *V. cholerae* O1 strains isolated from different countries [35–38]. The O139 *Vibrio* comprised three or more copies of the *ctxA* gene, and the chromosomal locations of these copies were unlike those of the El Tor or classical vibrios [39]. RFLP of *V. cholerae* O139 Bengal that resurged in Calcutta in 1996 were indistinguishable from the earlier strains by ribotyping, but the structure of the CTX genetic element was different [21, 40–42]. In most *V. cholerae* O139 strains isolated in China from 1993–1999 had two or more copies of CTX genetic elements and had extensive restriction patterns even in strains that belong to the same ribotype [43]. This finding suggests multiple origins of the O139 cholera epidemic or sporadic events. Similarly, *V. cholerae* O1 from Iranian cholera outbreak strains carried either three or two copies of the toxin genes [44].

Table 5.1 Localization and nature of CTX prophage alleles of *V. cholerae* O1

	Presence of CTXΦ		
Biotype	Chromosome I	Chromosome II	Type of *ctxB/rstR*
Classical	Yes	Yes	Classical/classical
El Tor	Yes	No	El Tor/El Tor
Mozambique variant	No	Yes	Classical/classical
El Tor variant	Yes	No	Classical/El Tor

Among newly emerged strains of *V. cholerae* O1 Inaba from India, presence of CTX prophage was detected in a single site of the chromosome with at least two RS elements [45]. Incidence of cholera in Mozambique was caused by an El Tor biotype *V. cholerae* O1 strain that carried a classical type (CTX^{class}) prophage [46, 47]. Genomic analysis of CTX prophage together with chromosomal phage integration sites showed that these strains carried two copies of prophages located in the small chromosome in tandem but the excised phage genome was deficient in replication and did not produce CTX^{class} virion [47]. The possible origin of these strains and the presence of the tandem repeat of the classical prophage in them implicate the presence of the classical CTX phage [46]. Table 5.1 gives an overall description about the localization and number of prophage alleles of *V. cholerae* O1.

Co-culture of a phage and *V. cholerae* or dilutions of phage-positive cholera stools in nutrient medium supported emergence of phage-resistant derivatives of the vibrios in vitro by losing their O1 antigen [48]. However, in vivo studies did not permit the selection and persistence of phage-resistant variants and the emerging variants and were thus unable to sustain the ongoing epidemic. This may be the reason why identification of new genetic variants is quite rare at the last phase of an outbreak.

When the DNA sequencing of *ctxB* from 45 *V. cholerae* O1 strains isolated in 29 countries over a period of 70 years were analyzed, 3 types of CT were identified [49]. The base changes correspond to an amino acid substitution in the B subunit of the CT. Genotype 1 was found in classical biotype worldwide and El Tor biotype strains associated with the US Gulf Coast. Genotype 2 was found in El Tor strains from Australia, and genotype 3 was found in El Tor biotype strains that represented seventh pandemic and the Latin American epidemics. The CT genotype 3, which predominated since early 1960s, has recently been replaced by genotype 1 in Bangladesh and India [50, 51]. The recent El Tor strains belonging to CT genotype 1 are found to be associated with several cholera outbreaks in India [52, 53]. Retrospective analysis with the *V. cholerae* O139 strains isolated during 1998–2005 in Bangladesh indicated prevalence of new CT genotypes such as 4, 5, and 6 [54]. Figure 5.1 summarizes many recent genomic changes in *V. cholerae* O1 and O139 strains.

5.5 Multilocus Enzyme Electrophoresis

Multilocus enzyme electrophoresis (MLEE) analysis (also known as zymovar analysis) compares genetic variation among a number of housekeeping genes and on the basis of electrophoretic mobility on starch gels. These variations are used to group

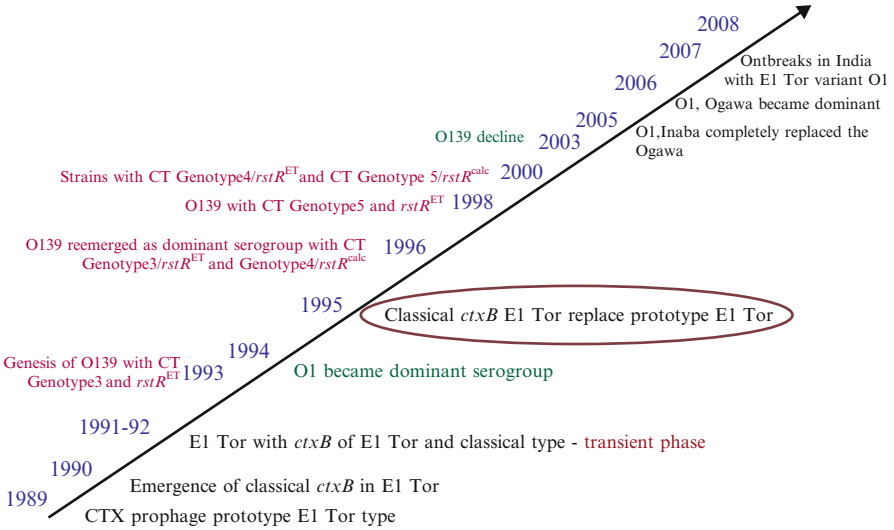

Fig. 5.1 Recorded major events in the evolution of *V. cholerae* O1 El Tor, El Tor variant and O139 CTX prophages

the *V. cholerae* into electrophoretic types (ETs) [38, 55]. With the use of 16 enzymes, Wachsmuth et al. [38]found existence of four distinct groups of toxigenic El Tor vibrios namely, the seventh pandemic, US Gulf coast, Australian and Latin American clones. With the *V. cholerae* non-O1 non-O139, classical and El Tor strains from America, Africa, Europe and Asia, Freitas et al. [56]have shown that the same zymovar may contain more than one serogroup and the South American epidemic strain differs from the seventh pandemic El Tor strain. However, the discriminatory power of MLEE is less but useful in distinguishing strains within a single outbreak.

5.6 Ribotyping

Ribotyping exploits the DNA polymorphism in the chromosomal regions of the highly conserved rRNA genes (*rrn*). *Escherichia coli* RNA is used as a universal probe to screen the restriction patterns of bacterial DNA for molecular typing purpose. In *V. cholerae*, *Bgl*I, is used as a discriminatory enzyme for ribotyping. The *rrn* operons and their flanking regions cause ribotype variation in *V. cholerae* O1 due to recombination in the *rrn* operons [57].

Though the seventh pandemic *V. cholerae* strains from Asia and Africa were clonal and belonged to a single ET, the ribotyping analysis showed that these strains were diverse and belonged to five different types [38]. Based on this observation, it was hypothesized that the observed differences were due to a higher mutation rate in

the DNA sequences flanking rRNA genes than in genes encoding the "housekeeping" enzymes studied using MLEE. A standardized scheme of 27 different *Bgl*I ribotypes and subtypes of *V. cholerae* O1 was developed on the basis of genetic analysis using strains collected over the past 60 years [58]. This analysis revealed 7 and 20 ribotypes among classical and El Tor biotypes, respectively. Six different patterns were found among seventh pandemic strains alone. Genetic variation and molecular evolution of sixth and seventh pandemic clone of *V. cholerae* O1 and its relationship with epidemiologically unassociated strains from different countries over 62 years (1931–1993) showed major differences in ribotypes [59]. Majority of seventh-pandemic isolates fall into two groups, the first present from 1961 to the 1993 and found only in Asia and the second arising in 1966 that had spread worldwide.

Ribotype analysis confirmed an association between epidemiologically related clinical isolates and the aquatic environment along with persistence of several clones of the *V. cholerae* O1 from the Australian environment [60]. RFLP of conserved rRNA showed that the El Tor strains isolated before the emergence of *V. cholerae* O139 belonged to four different ribotypes and the one that appeared after the emergence of O139 belonged to a single new ribotype [61]. This finding was fortified by the studies conducted independently in India and Thailand [62, 63]. These results provide evidence that the reemerged El Tor strains represent a new clone of El Tor distinctly different from the earlier clones, which were replaced by the O139 serogroup.

Analysis of *V. cholerae* O139 strains isolated in India and Bangladesh revealed four different ribotypes [22, 64]. Ribotypes I and II were shared by strains isolated from the epidemic outbreak during 1992–1993, ribotype III was represented by a single CT-negative O139 strain from Argentina, and majority of the reemerged strains isolated during 1995–1996 belonged to ribotype IV. These O139 strains may have emerged from similar serotype-specific genetic changes in more than one progenitor. In China, seven different ribotypes were recorded among *V. cholerae* O139 strains isolated between 1993 and 1999, suggesting the diversity of clones in phylogeny [43].

V. cholerae O1 strains isolated in Romania and the Republic of Moldavia (1977–1994) and Somalia (1998–1999) displaced different clones [17, 65]. The RFLP of *Bgl*I-digested DNA probed with five oligonucleotides targeting the conserved regions of 16S and 23S rRNA genes revealed a similar ribotype of *V. cholerae* El Tor strains isolated from outbreaks from different districts of Teheran, Iran [44]. The newly emerged *V. cholerae* O1 Inaba during 2004 and 2005 from different parts of India displayed different ribotypes [45, 66].

5.7 Pulse-Field Gel Electrophoresis

Pulse-field gel electrophoresis (PFGE) has proven to be highly effective molecular typing technique for different bacterial species. International PFGE typing protocol for *V. cholerae* was established for generation and submission of subtype patterns to

the data base ([67]; http://pulsenetinternational.org/). PFGE was shown to be useful for the identification of spread of specific clones in many cholera outbreak investigations. PFGE results suggested that there was no epidemiological relation among the strains of *V. cholerae* O1 isolated from indigenous cholera in Okinawa during 1994 [68]. With *V. cholerae* O1, 19 subtypes by *Not*I and *Sfi*I digested PFGE patterns were identified among Asian strains suggesting that the pulsotype variation is widely distributed in this region [69]. Based on the PFGE profiling, *V. cholerae* O1 Inaba strains isolated during 1998–1999 in Kolkata, India were different from the earlier Inaba strains isolated during 1989, but were similar to the prevailed *V. cholerae* O1 Ogawa strains, indicating that the Inaba strains may have the origin from Ogawa strains [70].

V. cholerae O1strains isolated from a cholera outbreak in Ahmadabad, India during 2000 resembled a PFGE pattern that was identified in Kolkata many years before, indicating the outbreak was caused by the prevailing clone. However, in the same outbreak, O139 differed in the PFGE patterns with O139 isolates reported during 1992 to 1997 in Calcutta [71]. Clonal analysis using PFGE with non-toxigenic *V. cholerae* O1 strains collected from the Port of Osaka, Japan during 1987–2001showed that there is no remarkable change in the fingerprint types [72]. PFGE results obtained from different outbreaks of *V. cholerae* O1 and O139 in Hong Kong and other epidemiologically unrelated strains showed combination of distinct and similar patterns [73]. In the same study, heterogeneous patterns were also identified among strains that were imported from other parts of Asia. *V. cholerae* O1 strains isolated from two cholera outbreaks (1999–2002) in Thailand demonstrated 17 PFGE banding patterns grouped into four Dice-coefficient clusters (PF-I to PF-IV). Prolonged appearance of the clone PF-II, the disappearance of clones PF-I and PF-III, and the emergence of new clones were reported [74]. The patterns of *V. cholerae* O1, El Tor reference strains from Australia, Peru, Romania, and the United States were different from the patterns of reference isolates from Asian countries, such as Bangladesh, India, and Thailand, indicating a close genetic relationship or clonal origin of the isolates in the same geographical region [74].

During 2004–2005, there was a serotype substitution from Ogawa to Inaba in India. New pulsotypes were identified from a cholera outbreak in Delhi during 2004 [66]. Majority of the Inaba isolates belong to "H1" pulsotype and one isolate is type "H," while the Ogawa isolates were mostly "H" pulsotype [45]. *V. cholerae* O1 Inaba collected during several cholera outbreaks throughout Iran during the summer of 2005 showed an identical ribotype and PFGE patterns in majority of the strains [9]. PFGE analysis of hybrid *V. cholerae* O1 strains isolated during 2004–2005 from cholera patients in Mozambique and Bangladesh showed five closely related patterns and had an El Tor lineage [75]. The restriction patterns grouped the hybrid strains from Mozambique into a separate cluster from Bangladeshi clinical and environmental strains. This study suggests that hybrid strains differed markedly from classical and El Tor biotypes. In Australia, sporadic cholera was due to indigenous *V. cholerae* O1 El Tor biotype from environmental sources. PFGE analysis revealed that the Australian environmental toxigenic *V. cholerae* O1 strains were more diverse from the non-toxigenic environmental O1 strains [29]. Since

there are no reference profiles in the pulsotyping scheme, it is difficult to correlate the enormous data that has been generated through many investigations.

5.8 DNA Sequence Based Typing Systems

5.8.1 Multilocus Sequence Typing

In multilocus sequence typing (MLST), the genetic variations at multiple housekeeping genes are directly indexed by nucleotide sequencing. This approach is suitable for database storage and software analysis and hence will address long-term epidemiological investigations when the bacterial populations are highly recombinant with large clonal complexes and have substantial time to diversify.

MLST with three housekeeping genes, *gyrB*, *pgm*, and *recA*, showed that there was clear clustering of epidemic *V. cholerae* O1 and O139 serogroups compared to the non-epidemic serogroups and MLST had better discriminatory ability than PFGE [76]. With the non-O1 and non-O139 strains, MLST revealed that were genetically diverse and clustered in lineages distinct from that of the epidemic strains [30]. The O139 strains also clustered in several lineages of the dendrogram generated from the matrix of allelic mismatches between the different genotypes [77]. In addition, the application of the Sawyer's test and split decomposition to detect intragenic recombination in the sequenced gene fragments did not indicate the existence of recombination in the tested strains. Using MLST with 26 housekeeping genes, Salim et al. [78]showed that the US Gulf strains, Australian strains and some of the strains similar to the El Tor strains belong to the seventh pandemic clone, whereas the sixth pandemic strains were separated in this analysis.

5.8.2 Variable Number of Tandem Repeat Loci

In the bacterial genome, repetitive DNA contains monomeric sequences (repeat loci) frequently and arranged in a head-to-tail configuration. These DNA regions are known as variable number of tandem repeat (VNTR) that are catalogued on the basis of their repeat unit sizes (ranges from few nucleotide to more than 100 bp). The repeat loci in nearly all the VNTR targets are highly conserved and hence the discrimination power is more compared to that of MLST.

To explore the relatedness of O139 strains isolated from cholera patients in India, Garg et al. [79] analyzed nine sequenced loci and found 64 novel alleles distributed among 51 sequence types. Lateral gene transfer (LGT) events produced three times the number of nucleotide changes compared to mutation [79]. In contrast to the traditional concept of epidemic spread of a homogeneous clone, the establishment of variant alleles generated by LGT during the rapid expansion of a clonal bacterial

population may be a paradigm in infections and epidemics. Ghosh et al. [80] evaluated genetic relationships of *V. cholerae* isolates collected between 1992 and 2007 from different states in India by analyzing five VNTR loci. In this study, it was found that each VNTR locus was highly variable, with 5–19 alleles. E-burst analysis revealed four large groups of genetically related strains. Two groups contained genotypes with O139 serogroup and the other two groups with O1strains. Using VNTR analysis, it is possible to track the spread of specific genotypes across time and space. It was observed that the minimal overlap in VNTR patterns between the two Bangladeshi communities was consistent and it was concluded that the small outbreaks of cholera were mainly from local sources [81].

5.9 Fingerprinting of Virulence Genes

It is a general concept that the pathogenic *V. cholerae* are derived from environmental non-pathogenic strains. Potential precursors of new pathogenic strains might require a combination of genes for both ecological fitness and virulence to attain epidemiological predominance. To understand the evolution of pathogenic *V. cholerae* and identify potential precursors of new pathogenic strains, Rahman et al. [27] analyzed environmental or clinical strains for the presence of diverse genes involved in virulence or ecological fitness. This study has shown that 3.9% carried the pathogenicity island encoding TCP and CT. Few strains carried the TCP island alone and were susceptible to transduction with CTX phage. Prevalence of putative accessory virulence genes (*mshA, hlyA*, and *rtx*) both in toxigenic and non-toxigenic strains of *V. cholerae* support more recent assumption that these genes imparts increased environmental fitness and the epidemiological success of the strains [27].

The evolutionary relationships and multilocus virulence gene profiles of *V. cholerae* isolates indicate that consecutive pandemic strains arose from a common O1 serogroup progenitor through the successive acquisition of new virulence regions. Comparative sequence analysis of malate dehydrogenase gene (*mdh*) revealed that *V. cholerae* O1 and O139 serogroups belonged to the same clonal lineage. Single-strand conformational polymorphism (SSCP) analysis of these O1 and O139 strains at chaperonin (groE)L confirmed the presence of an epidemic clonal complex [26].

5.10 Evidence Showing Intercontinental Spread of *V. cholerae* O1

Several molecular techniques were used for the detection of *V. cholerae* clones that has been spread from one geographical region to the other. With ribotyping and PFGE, spread of a distinct genotype of *V. cholerae* O1that appeared in Calcutta, India was detected from cholera cases in Guinea-Bissau from 1993 to 1996 [82]. Molecular epidemiological findings confirm that the epidemic Ukrainian strains are most

closely related to seventh pandemic *V. cholerae* O1 strains from Asia and support a hypothesis that the Ukrainian epidemic during 1994–1995 was caused by toxigenic environmental strains surviving since 1991 [83].

After a decade of absence, *V. cholerae* O1 resurfaced in Italy and Albania during 1994. Ribotype, RAPD, and PFGE patterns indicated that the 1994 isolates belonged to the Asian clone, which emerged in 1990 [84]. The Mozambique *V. cholerae* O1 strains that caused a huge outbreak in 2004 had phenotypic traits of both classical and El Tor biotypes. Interestingly, these strains harbored a CTX prophage in the smaller chromosome similar to that of classical biotype. Retrospective studies conducted in India demonstrated that O1 strains isolated in Kolkata during 1992 were phenotypically identified as El Tor biotype but the *ctxB* was classical genotype [85]. *V. cholerae* O1 Mozambique variant shared most of its genes with the typical El Tor strain N16961 but did not carry the TLC gene cluster, and RS1element adjacent to the CTX prophage [47]. This data further support the hypothesis that the Mozambique strain having evolved from a progenitor similar to the seventh pandemic strain, involving multiple recombination along with origination of El Tor strains carrying the classical CTX prophage. Furthermore, the Kolkata strains exhibited an identical ribotype (RI) to that of the Mozambique variant and the *Not*I pulsotype analysis indicated that the Kolkata O1strains and the Mozambique variant belonged to closely related clones. Considering the chronological events, and the typical identity at the phenotypic and the genotypic level, Chatterjee et al. [85] proposed that one of the 1992 Kolkata O1 strains might have been the progenitor for Mozambique variant O1 strains.

5.11 Quorum-Sensing Systems

During interepidemic periods of cholera, *V. cholerae* survive in aquatic habitats. Recent studies reveal that quorum-sensing systems (QSS) help the vibrios to regulate various cellular functions, pathogenesis, biofilm formation, and protease production [86]. Quorum sensing systems in geographically diverse *V. cholerae* from epidemic-causing O1and O139 as well as non-O1/non-O139 and environmental strains revealed unexpectedly high rate of dysfunctional components and variations in quorum-sensing systems due to environmental selective pressures [86]. The use of QSS as a typing tool may provide several information regarding survival and proliferation of vibrios and subsequent causative agent of cholera in adjacent areas.

5.12 Microarray Analysis

Whole genome sequence of the seventh pandemic El Tor strain N16961 has provided an important tool for addressing questions about the evolution of *V. cholerae* as a human pathogen and environmental organism. To understand *V. cholerae*

genome, Dziejman et al. [87] constructed a genomic microarray that displayed over 93% of the predicted genes of the strain N16961as spotted features. High degree of conservation among the strains tested was detected with hybridization of labeled genomic DNA. Genes unique to all pandemic strains as well as genes specific to seventh pandemic El Tor and related O139 serogroup strains were also identified. It was assumed that the odd genes may encode gain-of-function traits specifically associated with displacement of the preexisting classical biotype and might promote the establishment of endemic disease in cholera-free geographical areas.

Pang et al. [88] investigated the genomic diversity of toxigenic and non-toxigenic O1 and O139 strains by comparative genomic microarray hybridization against the genome of El Tor strain N16961. High phylogenetic diversity in non-toxigenic O1 and O139 strains was detected and most of the genes absent from non-toxigenic strains are clustered together in the N16961 genome. Additionally, sequence variation in virulence-related genes was found in non-toxigenic El Tor strains. The small chromosome of *V. cholerae* is quite conservative outside of the superintegron region (SIR). However, the SIR showed pronounced genetic divergence in both toxigenic and non-toxigenic strains. Comparative genomic microarray analysis of four pathogenic *V. cholerae* non-O1, non-O139 strains indicated that these strains are quite divergent from O1 and O139 strains [89]. In addition, a pathogenic non-O1, non-O139 strain carried a type III secretion system (TTSS). The genes for this *V. cholerae* TTSS system appear to be present in many clinical and environmental non-O1, non-O139 strains, including at least one clone that is globally distributed.

5.13 Whole Genome Approach

To understand the origin and relationships of the pandemic clones, Feng et al. [90] did sequence analysis of genomes of a 1937 pre-pandemic strain and a sixth pandemic isolates, and compared them with the published seventh pandemic strain (N16961). Many mutational than recombination events were detected as much as 100-fold higher in seventh pandemic strain compared to the pre-pandemic one. It was assumed that these pandemic strains have gained pandemic potential independently with 29 insertions or deletions of genes in one or more genes. There were also substantial changes in the major integron, attributed to gain of individual cassettes including copying from within, or loss of blocks of gene cassettes. The genome-based phylogenetic analysis with sequences of *V. cholerae* strains isolated from a variety of sources over the past 98 years revealed 12 distinct lineages, of which one comprises of classical and El Tor biotypes [91]. It was affirmed that transition from sixth to seventh pandemic strains as a genetic shift and transition among clones during the present pandemic period as drift of clones with varying composition of laterally transferred genomic islands, resulting in emergence of variants [91].

5.14 Epilogue

Research on cholera is always inundated with several challenges especially when the pathogenic clones carry diverse combinations of phenotypic and genotypic properties. Newer concepts are often being proposed for dynamicity of the pathogen supported with different genomic analyses. For example coculture of a phage and *V. cholerae* or dilutions of phage-positive cholera stools in nutrient medium supported emergence of phage-resistant derivatives of the vibrios in vitro by losing their O1 antigen [48]. However, in vivo studies did not permit the selection and persistence of phage-resistant variants and the emerging variants and thus unable to sustain the ongoing epidemic. This may be the reason why identification of new genetic variants is quite rare during the last phase of an outbreak. It was also hypothesized that new genetic variants emerge due to up take of extracellular DNA by the naturally competent bacteria in the aquatic environments [92]. The genome of *V. cholerae* is well understood and several molecular typing methods exist for the detection of subtle changes. Multidisciplinary genomic analysis alone may not be the right direction as we might overlook the simple nucleotide changes that may influence the disease outcome. For example simple mutational changes such as the one detected in the *ctxB* and emergence of new CT genotypes of *V. cholerae* was shown to manifest considerable impact on epidemiology of cholera globally [93, 94]. As mentioned in several sections of this chapter, many molecular typing techniques are now available and are used in different situations and times. However, the use of appropriate method is left to the investigator considering the gravity of the problem, experimental cost, and existing facilities.

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Chapter 6
Molecular Typing of *Clostridium difficile*

Andrej Weintraub and Carl Erik Nord

6.1 Introduction

Clostridium difficile is a Gram-positive, sporulating anaerobic rod that causes diarrheal illness generally called *Clostridium difficile* infection (CDI). CDI may manifest as a range of mild, self-limiting diarrhea to life threatening syndromes such as pseudomembranous colitis and toxic megacolon. *C. difficile* is considered the main etiological agent of antibiotic associated diarrhea and is the most common cause of nosocomial diarrheal disease [1]. The major virulence factors in *C. difficile* associated with the CDI, are the toxins A and B. Toxin A is an enterotoxin (TcdA, 308 kDa) and toxin B, a cytotoxin (TcdB, 270 kDa). Most of the virulent strains produce both toxins. However, pathogenic strains producing only toxin B have been identified [2]. The genes for the toxin A and B are located on the Pathogenicity Island called PaLoc. During the last decade, a new epidemic strain of *C. difficile* has emerged in Canada, USA, and Europe causing major outbreaks in hospitals. This particular strain was shown to produce an additional toxin, binary toxin (CDT). The genes of the binary toxin are located outside the PaLoc loci.

Since the discovery of *C. difficile* as the causative agent of diarrhea and pseudomembranous colitis in the late 1970s, several diagnostic methods have been developed both for the clinical diagnosis as well as for epidemiological studies. The typing methods can be divided into two major categories: phenotypic and genotypic. The phenotypic methods are mainly focused on the detection of the toxins as well as colony morphology on special selective media. The genotypic methods are mainly focused on the molecular genetic profile of the isolates. For diagnostic purposes, phenotypic methods are widely used especially the culture of the microorganism and toxin detection, i.e., toxigenic culture. This method is still considered as

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the "Gold Standard" in laboratory diagnosis of CDI. However, genetic methods mainly based on detection of the toxin genes are emerging as a complement to the time- and labor-consuming phenotyping methods.

In order to study the epidemiology of CDI, it is essential that the method has (1) high discriminatory power, (2) high typeability, and (3) high reproducibility. The epidemiological typing of *C. difficile* is important especially during minor as well as major outbreaks in hospital(s) and to evaluate the possible patient-to-patient transmission. Since the rate of recurrences of CDI is estimated to be 20–30%, the molecular typing of *C. difficile* strains may distinguish between relapses due to the same strain or reinfection due to a different strain.

6.2 Molecular Methods for Laboratory Diagnosis

There are a number of commercially available molecular methods for detection of *C. difficile* in clinical samples, i.e., feces. The methods are based on PCR detecting either the genes for the toxin(s) or a conservative region in the PaLoc loci.

6.2.1 Cepheid Xpert™ C. difficile Assay

The Cepheid Xpert™ *C. difficile* assay is a multiplex real-time PCR method for the detection of toxigenic *C. difficile* strains. The Cepheid Xpert™ *C. difficile* assay detects the genes for toxin B (*tcdB*), binary toxin (*ctdA/B*) as well as the *tcdC* deletion nt 117 that is present in some of the recently identified epidemic strains. This deletion results in an inactive *tcdC* product, which is a negative regulator of the *tcdA* and *tcdB* genes resulting in an increased production of toxin A and B. The Cepheid Xpert™ *C. difficile* assay is user friendly and the total turnaround time is <60 min. Evaluation of the assay shows that the sensitivity, specificity, and positive and negative predictive values of the Xpert assay were 93.5–100%, 93–96.7%, 72.3–90.5%, and 98.8–100%, respectively, as compared to the cell cytotoxicity neutralization assay (CCNA) and/or toxigenic culture [3–6]. With the results available within one hour and with the high specificity and sensitivity, the assay provides prompt and precise clinical laboratory diagnosis. It would be of advantage if this assay could also detect the toxin A gene (*tcdA*).

6.2.2 Loop-Mediated Isothermal Amplification Assay

Loop mediated isothermal amplification (LAMP) is an innovative gene amplification. The whole procedure is very simple and rapid wherein the amplification can be completed in less than 60 min under isothermal conditions. A set of six primers spanning a distinct sequence of a highly conserved part of the Toxin A gene (*tcdA*) are used. Adding the sample to a tube containing all the reagents makes the assay simple and

easy to use. Gene amplification products are detected by real-time monitoring in a turbidimeter. The rapid amplification, simple operation and easy detection make the LAMP technique for detection of *C. difficile* in clinical samples an attractive molecular method for detection of CDI in clinical laboratories. There is one publication available evaluating the assay in clinical setting. Analyses of 272 samples by the LAMP *C. difficile* assay with the CCNA and/or toxigenic culture as comparator revealed a sensitivity of 98%, specificity of 98%; PPV of 92% and NPV of 99% [7].

6.2.3 BD GeneOhm Cdiff Assay

The basis for the BD GeneOhm Cdiff assay is a RT-PCR detection of toxin B (*tcdB)* gene in the clinical sample. The assay includes lysis of the sample and DNA extraction step followed by a RT-PCR analysis using a Smartcycler (Cepheid). The turnaround time for each sample is <2 h. The assay has been evaluated in several publications and compared to the CCNA and/or toxigenic culture, the overall sensitivity, specificity, PPV, and NPV varied from 83.6–92.2%, 94–100%, 68–100%, and 97–98.7%, respectively [8–10].

6.2.4 Conclusions: Molecular Methods for Laboratory Diagnosis

There are three commercial assays for clinical laboratory diagnosis of CDI. They have been approved by the FDA for use in North America. All the assays are rapid and relatively easy to use. One of the problems in evaluation of molecular assays is the use of a comparative method. The "golden standard" for *C. difficile* diagnostics is the toxigenic culture using the cell cytotoxin neutralization assay. In addition, the selection of the samples may influence the outcome of the comparison. The samples to be evaluated should be collected from patients suspected to have CDI. The sample should consist of a loose stool. An additional problem that may influence the negative and positive predictive values is the prevalence of the disease at the time and the location where the evaluation is performed. If the prevalence of CDI is relatively low (<10%), the positive predictive value of different assays may be lower compared to settings where the prevalence is high [11].

6.3 Molecular Methods for Epidemiological Characterization

Several different methods for molecular typing of *C. difficile* isolates are described in the literature. Some utilize the whole genome and rare cutting restriction enzymes (REA, RFLP, PFGE, AFLP), while others are based on amplification of either specific regions of the genomic DNA or specific genes in the DNA (PCR-ribotyping, RAPD and MLVA). In addition a PCR-based method in combination with sequencing

has been developed for the classification of *C. difficile* [MLST, toxinotyping, and surface-layer protein A sequence typing (*slpA*ST)]. All the methods are laborious and possess advantages and disadvantages (Table 6.1). They are very useful for epidemiological surveillance but not for clinical laboratory diagnostics.

6.3.1 Restriction Enzyme Analysis

The restriction fragment analysis (REA) method utilizes the whole genomic DNA, which is digested by a rare cutting restriction enzyme and analyzed by gel electrophoresis. The banding pattern can be very complex and comparisons between different laboratories difficult. The first description of this method for the classification of *C. difficile* was reported in 1987 and the enzymes *Hin*dIII and *Xba*I were used [12]. Other restriction enzymes have been used with good results [13, 14]. Clabots et al. analyzed almost 2,000 *C. difficile* isolates from various sources using the REA method with the *Hin*dIII restriction enzyme. The collection resulted in 206 unique REA types and was grouped into 75 groups [13]. In a more recent study, Kilgore et al. investigated 42 *C. difficile* isolates by different molecular methods. Using REA, the collection was divided in 10 REA types and 27 subtypes [15]. REA is a highly discriminatory and reproducible technique for epidemiological characterization of *C. difficile* strains. However, the method is labor-intensive and the evaluation may be difficult with complex banding patterns. In addition, exchange of results between laboratories and comparison of the results is very difficult. The REA method is used in some laboratories in North America.

6.3.2 Restriction Fragment Length Polymorphism

The restriction fragment length polymorphism (RFLP) is rather similar to the above-described REA method. The initial step is a digestion of the whole genomic DNA with the *Hin*dIII restriction enzyme and gel electrophoresis followed by Southern blotting. Labelled nucleic acid probes are used to highlight specific restriction site heterogeneity. The first description of the RFLP method for the characterization of *C. difficile* was published in 1991 by Bowman et al. In this study, commercially available *Escherichia coli* ribosomal ribonucleic acid (rRNA) as probe material was used. Probe labeling, hybridization and detection was performed using the Enhanced Chemiluminescence gene detection system [16]. The method was easy to perform with relative good discriminatory power. The RFLP method has also been used with other labelled probe such as the eubacterial 16S rRNA and proved to give a simpler and more discriminative pattern [17]. A comparison between REA and RFLP using the same restriction enzyme, *Hin*dIII, showed that REA is much more discriminatory that RFLP. One hundred and sixteen

Table 6.1 Features of different molecular typing methods for *Clostridium difficile*

Method	Target	Method	Discriminatory power	Reproducibility	Performance	Inter-laboratory exchange
REA	Whole genome	Restriction	Low	Low	Low	None
RFLP	Whole genome	Restriction	Low	Low	Low	None
PFGE	Whole genome	Restriction	High	High	Low	Low
AFLP	Whole genome	Restriction	Intermediate	Intermediate	Low	Low
RAPD	Whole genome	Random PCR primers, amplification	Intermediate	Low	Intermediate	None
PCR-ribotyping	16S–23S intergenic spacer region	Specific primers, amplification	Intermediate	High	High	High
Toxinotyping	Toxin A, B and binary toxin genes	Specific primers, amplification	Low	High	High	Intermediate
MLST	Seven housekeeping genes and ten virulence associated genes	Specific primers, amplification and sequencing	Intermediate	High	High	High
MLVA	Whole genome, tandem repeats	PCR amplification	High	High	High	High
*slpA*ST	Specific genes	Specific primers, amplification and sequencing	Low	Intermediate	High	Intermediate

Abbreviations: *REA* restriction enzyme analysis, *RFLP* restriction fragment length polymorphism, *RAPD* random amplified polymorphic DNA, *PFGE* pulsed-field gel electrophoresis, *AFLP* amplified fragment length polymorphism, *MLST* multi-locus sequence typing, *MLVA* multiple-locus variable number tandem repeat analysis, *slpAST* surface-layer protein A sequence typing

isolates were studied and the results showed that using REA, 34 types could be distinguished. Using RFLP, with the same collection of isolates, the corresponding figure was 6 types [18].

6.3.3 Random Amplified Polymorphic DNA

The random amplified polymorphic DNA (RAPD) method for characterization of *C. difficile* was first described in 1993 by Barbut et al. [19]. This is a PCR based and short oligonucleotide primers ~10 bp with an arbitrary sequence are used. The profiles observed after electrophoretic separation were able to distinguish 20 reference *C. difficile* strains. In another study, Chachaty et al., used 3 different 10-bp oligonucleotides and analyzed 30 unrelated *C. difficile* strains. The isolates could be divided into 25 RAPD types suggesting a good discriminatory power [20]. The method is simple to use and can give good results in an initial screening of isolates suspected to cause outbreaks. However, comparison of the gel electrophoresis patterns can be cumbersome to evaluate and inter-laboratory exchange of the electrophoretic banding patterns rather difficult.

6.3.4 PCR-Ribotyping

The PCR ribotyping method is based on amplification of an intergenic spacer region between the 16S and 23S rRNA genes and the use for characterization of *C. difficile* was first described in 1993 [21]. In *C. difficile* multiple copies of the rRNA genes that also vary in length are present. A single primer pair can be used in a PCR reaction, which usually yields a pattern of fragments of 200–700 bp. Usually, the bands are separated by either agarose or polyacrylamide gel electrophoresis. There are many publications describing the PCR-ribotyping method [21–28]. Currently the method described by O'Neill [24] is mostly used. Recently, the mechanism behind the variation of the 16S–23S rRNA intergenic spacer region has been published [29]. A PCR-ribotype is defined as a group of strains that produce an identical band pattern. A single band difference warrants a new ribotype. A standardization of the PCR-ribotyping method has been done at the Anaerobe Reference Unit, Cardiff, UK. More than 10,000 *C. difficile* isolates from different sources have been analyzed and a library of more than 200 ribotypes has been constructed. The nomenclature of the PCR-ribotypes is by a three-digit number starting from 001. At present, the PCR-ribotyping method is the most common molecular method for characterization of *C. difficile* strains in Europe. However, a correct international PCR-ribotype can only be assigned when compared with reference strain(s). In many laboratories, a local nomenclature is used making inter-laboratory comparisons difficult. This problem may be circumvented using a capillary gel electrophoresis as described recently by Indra et al., [30]. The authors analyzed 146 *C. difficile* isolates by PCR-ribotyping using conventional gel electrophoresis and compared the results with capillary gel

electrophoresis. The method seems to be more discriminatory than the conventional agarose separation. The capillary gel electrophoresis was able to divide 24 isolates belonging to PCR ribotype type 014 into seven subgroups. A Web-based software program (http://webribo.ages.at) has been developed. This may, in the future, overcome the problems with inter-laboratory comparison and increases the possibility for further standardization of the PCR-ribotyping method.

6.3.5 Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis is the standard molecular biological method in bacteriology. It is used for characterization of a variety of bacterial species. PFGE was one of the first molecular typing methods used for *C. difficile*. PFGE is still the standard method for molecular typing in North America [31]. In PFGE the whole genome is digested using restriction enzymes such as *Sma*I or *Sac*II [31–35].

Using the *Sma*I restriction enzyme in PFGE results in 7–15 fragments (range 10–1,100 kbp), while the *Sac*II results in 10–20 fragments. When two isolates show ≤80% similarity, they are considered to belong to the same pulsotype. In North America the isolates are designed as NAP and numerical number, i.e., NAP1 (North American Pulsotype 1). The advantage of PFGE is a high discriminatory power; however, the disadvantages are several. The method is time (4–5 days) and labor-demanding. There are no standard protocols allowing easy inter-laboratory comparisons.

6.3.6 Toxinotyping

Toxinotyping is an RFLP-PCR based method for differentiating *C. difficile* strains based on the detection of polymorphism in the part of the genome where the *Pathogenicity locus* (PaLoc) is located. The PaLoc in *C. difficile* contains the genes for the toxins A and B as well as the regulatory genes for the expression of the toxins. In the toxinotyping, six regions of the PaLoc are amplified using specific primers for each. The regions are called A1–A3 and B1–B3. The amplicons of regions B1 and A3 are then digested with restriction enzymes. For the toxinotyping, region B1 is digested with two restriction enzymes, *Acc*I and *Hinc*II (B1). The A3 amplified region is cut with only one restriction enzyme, *Eco*RI [36–39]. The toxinotypes are designated by Roman numerals (I–XXXI) and 31 different types have been recognized until now (http://www.mf.uni-mb.si/mikro/tox) (Table 6.2).

6.3.7 Amplified Fragment Length Polymorphism

In Amplified Fragment Length Polymorphism (AFLP), a specific subfraction of multiple genomic restriction fragments is amplified by PCR, finally resulting in high-resolution subgenomic fingerprints. The AFLP method uses restriction, ligation,

Table 6.2 *Clostridium difficile* toxinotypes

Toxin	Toxinotype
$A^+B^+CDT^+$	IIIa-c, IV, V, VI, VII, IX, XIV, XV, XXII, XXIII, XXIV, XXV, XXVIII
$A^+B^+CDT^-$	0, I, II, XII, XIII, XVIII, XIX, XX, XXI, XXVI, XXVII, XXIX
$B^+A^-CDT^+$	X, V-like, XVI, XVII, XXX, XXXI
$B^+A^-CDT^-$	VIII
$A^-B^-CDT^+$	XIa, XIb
$A^-B^-CDT^-$	XI

A—Toxin A; B—Toxin B; CDT—Binary toxin

and selective amplification on the whole genome. Differentiation can be made due to variation per type in restriction site mutations, mutations in the sequences adjacent to the restriction sites and complementarity to the selective primer extensions, and insertions and deletions within the amplified fragments. For *C. difficile* the method was first described in 2002 [40]. The authors compared PFGE and AFLP using 30 clinical *C. difficile* isolates. AFLP analysis yielded high resolution and highly reproducible DNA fingerprinting patterns from which the epidemiological relatedness among the isolates could easily be determined. AFLP results could be readily obtained within 24 h, whereas 3–4 days were routinely required to complete the lengthy PFGE protocol. AFLP clearly proved to be a much more fail-safe fingerprinting method for *C. difficile* isolates, especially for those isolates for which a standard PFGE procedure yielded inconclusive results due to DNA degradation [40]. After the initial publication, AFLP was used in few other studies mainly to compare the technique with other molecular typing methods for *C. difficile* [15, 41].

6.3.8 Multi-Locus Sequence Typing

MLST characterizes multi-locus genotypes of bacterial isolates by using 400- to 500-bp intragenic sequences of several (generally seven) housekeeping genes. MLST presents a high sensitivity due to its ability to detect neutral genetic variations. The DNA sequences are unambiguous and comparable between different laboratories and can be stored in a shared central database to provide a broader resource for epidemiological studies. In addition, evolutionary genetics studies can be performed, since MLST describes variations affecting housekeeping genes. The first description of the use of MLST for the characterization of *C. difficile* was described by Lemee et. al. in 2004 [42]. Among 72 isolates from various origins, 62 PCR ribotypes and 34 sequence types (STs) could be discriminated. In a dendrogram representing the relationships between the STs, three divergent lineages could be recognized, of which one strictly contained toxin A−/B+ strains. A further development of the MLST including several virulence-associated genes has been described [43]. Toxin A−/B+ strains belonged to a homogeneous lineage; however,

a fourth lineage could be characterized in contrast to the method based on only housekeeping genes. A comparison of MLST with all other, above described, techniques showed that MLST is more discriminatory than AFLP but less than MLVA, REA, PFGE, and PCR-ribotyping [15].

6.3.9 Multiple-Locus Variable Number Tandem Repeat Analysis

The basis for Multi-locus Variable number tandem repeat Analysis (MLVA) is the fact that the bacterial genome contains a variable number of tandem repeats (VNTR). The repeats vary in complexity, size and location and may occur clustered or dispersed. MLVA for *C. difficile* was first described by Marsh et al. [44]. The authors used automated sequence detection and manual determination of the number of the tandem repeats per locus. The method was compared to REA (see Sect. 3.1 in this chapter) and it was shown that the MLVA clustered strains of the same REA type and discriminated different REA types. The method was further developed using tandem repeats of 2–9 bp and analyzed by multicolored capillary electrophoresis [45]. The MLVA was highly reproducible and showed the highest discriminatory power as compared to all other molecular methods for typing of *C. difficile* isolates [15]. The MLVA has been evaluated in several studies and compared with different techniques and showed to have a very high discriminatory power [45–49].

6.3.10 Surface-Layer Protein A Sequence Typing

Analysis of surface-layer proteins in *C. difficile* has also been used for typing of the species. The low-molecular mass peptide of the surface-layer protein varies among *C. difficile* isolates [50, 51], The variable region in the surface-layer protein A gene (*slpA*) have been used in the typing of *C. difficile* isolates by a combination of PCR-RFLP and sequencing [52, 53]. Recently, the slpA sequence typing was applied successfully to direct typing of *C. difficile* from DNA extracted directly from stool [54]. As the direct typing method depends on the variability of the *slpA* gene, three sets of primers for the second PCR were used to amplify the variable region of the gene. The method could be valuable for detecting epidemiologically important strains.

6.3.11 Conclusions: Molecular Methods for Epidemiological Characterization

All the above described epidemiological characterization methods have some advantages and disadvantages. Choosing a method will depend on the laboratory set-up and on the purpose of the epidemiological study. The important facts that

need to be taken into account are: (1) type-ability; (2) discriminatory power; (3) stability and (4) reproducibility. Depending on the method, it can be used for inter-laboratory exchange of the result or only locally at a particular laboratory. The methods of highest degree of inter-laboratory exchange possibility are the PCR-ribotyping, MLST and MLVA. The methods of highest discriminatory power are PFGE and MLVA. The best reproducibility is achieved using the PFGE, PCR-ribotyping, toxinotyping, MLST and MLVA.

6.4 Conclusions

Molecular typing of *C. difficile* can be divided in to two areas, one for diagnostic and one for epidemiological purposes. For clinical diagnostics, the methods are based on the detection of the genes for the toxins. The methods described in this chapter are all commercially available and have very similar performance regarding sensitivity and specificity. The choice of method will depend on the logistics in the laboratory, the hands-on time, cost for the equipment, as well as on the price per test. The different epidemiological molecular methods all have advantages and disadvantages. The discriminatory power differs between the method and the choice will depend on the individual laboratory interest. Some of the methods are more standardized and the results easy exchangeable between laboratories. Others are more "in-house" and useful in a particular laboratory. Some are easy to perform and others require sophisticated equipment and skilled staff. The important issues to consider, regardless of which method is used, are (1) type ability, (2) reproducibility, (3) stability, and (4) discriminatory power.

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Chapter 7
Oral and Intestinal *Bacteroidetes*

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7.1 General Introduction

Bacteroidetes are a phylum of bacteria comprising three classes: *Bacteroides*, *Flavobacteria*, and *Sphingobacteria* representing three families and two genera: *Bacteroidaceae, Flavobacteriaceae, Flexibacteriaceae, Rhodothermus,* and *Sphingobacterium*. However, as the taxonomy is in a constant flux, the reader is asked to update the composition of *Bacteroidetes* whenever relevant (see: www.bacterio.cict.fr). We focus on the class *Bacteroides* which consists of the genera *Bacteroides*, *Parabacteroides*, *Porphyromonas,* and *Prevotella* and all four are discussed here but using and explaining different typing methods, exemplarily. As originally isolated from Bacteroides-Bile-Esculin (BBE) agar -and thus traditionally coinvestigated with *Bacteroides*, the urease- and catalase-positive, nitrate-reducing anaerobic Gram-negative species *Bilophila wadsworthia*, even though a member of deltaproteobacteria (*Desulfovibrionaceae*), is also subjected here.

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7.2 The Genus *Bacteroides*

The genus *Bacteroides* has undergone many revisions in the past 20 years. The list of approved species within the genus *Bacteroides* changes frequently, and keeping up with all relevant taxonomic revisions is quite a challenge. However these changes are of importance both to clinicians and to clinical microbiologists, since taxonomic placement can be an indicator of virulence potential or antimicrobial resistance. In 1988–1989, the species within the former "*Bacteroides*"-group were restricted to members of the *B. fragilis* group [1], and most of the other clinically relevant species became placed in the genus *Porphyromonas* or *Prevotella* [2]. More recently, hosts of other genera have been described for *Bacteroides* species, including, among others, *Alistipes, Dialister, Megamonas, Mitsuokella, Odoribacter, Rikenella, Sebaldella, Tannerella,* and *Tissierella*. Often by using culture-independent approaches such as 16S rRNA gene sequencing, a variety of new species have added to the total number of *Bacteroides* species (now >38) [3]. In recent years, several species have been added to the genus *Bacteroides*, including *Bacteroides nordii*, *Bacteroides salyersai*, *Bacteroides plebeius,* and *Bacteroides coprocola* from human feces, and *Bacteroides massiliensis* isolated from the blood culture of a newborn. The new species, *B. goldsteinii* as well as *B. distasonis* and *B. merdae*, were only temporarily *Bacteroides* species but then moved to the new genus *Parabacteroides* [3].

As *Bacteroides* (*B. fragilis*) may have both a good and bad nature, molecular typing aims to differentiate between physiological and pathogenic strains. The pathogenicity of *B. fragilis* is related to the "*B. fragilis* pathogenicity island or BfPAI," producing the enterotoxin, which is a zinc metalloprotease [4]. For historical background, In the mid-1980s it was recognized that some *B. fragilis* strains produce an enterotoxin (ET) that can cause acute diarrhea in humans, young lambs, calves, pigs, and foals [5]. Later, enterotoxigenic *B. fragilis* (ETBF) strains have also been isolated from the feces of children with diarrhea [6, 7]. Kato et al. [8] showed that *B. fragilis* blood culture isolates were more likely to be ETBF and suggested that ET-positive strains are more virulent than ET-negative strains. The corresponding enterotoxin gene (*bft*) was cloned, sequenced and identified as producing a zinc metalloproteinase with the size of 44.4 kDa [4]. The *bft* gene is located in a 6 kb genetic element termed the *B. fragilis* pathogenicity island (BfPAI). In our studies [9] (and unpublished data), it was determined that the incidence of ETBF in different clinical isolates was 11–23%. The prevalence of ETBF among blood culture isolates (23%) was higher than from other specimen, especially the physiological gut isolates. Appendicitis and peritonitis are typical clinical *Bacteroides*-related cases but which often demonstrate mixed infections with *Enterobacteriaceae* (not subjected here) and *Bilophila* [9].

7.2.1 The Genus Bilophila

Bilophila (with a single species: *B. wadsworthia*) was first described by Baron et al. as an asaccharolytic, Gram-negative, bile-resistant, strong catalase-positive bacillus that is often urease positive (approximately 75% of strains) and able to reduce

nitrate to nitrite. The G+C-content is 39–40 mol% [10, 11]. Growth is stimulated by taurine, a cysteine derivative and major organic solute in humans, which it uses as a source of sulphite and as a terminal acceptor for electron transport [12]. Phylogenetically, the genus *Bilophila* is located in the deltaproteobacteria (*Desulfovibrionaceae*). Several virulence factors such as abscess formation, endotoxin, cytotoxicity, and adherence as well as outer membrane proteins were determined in *B. wadsworthia* [3, 12].

7.2.2 Bacteroides: Methods

7.2.2.1 Phenotypic Identification of Gram-Negative Anaerobic Saccharolytic Rods

Molecular typing can never stand alone but needs state-of-the-art conventional identification as a precondition before being performed. The traditional method for identification and classification of anaerobic bacteria uses carbohydrate fermentation and other biochemical tests in combination with metabolic end-product analysis by gas chromatography and, taken together, still provides the "Gold Standard" for identification. The biochemical scheme for identification of *Bacteroides* species and *B. wadsworthia* has been described previously and updated in detail [13]. In brief, prereduced, anaerobically sterilized (PRAS) biochemicals are used to test the fermentation of arabinose, rhamnose, trehalose, salicin, sucrose, xylan, the hydrolysis of esculin, and the production of indole and catalase. Bile resistance is usually determined by growth in PRAS peptone/yeast broth containing 20% bile. In addition, key reactions of the RapID ANA II systems are used. In case of *Bacteroides* species gas chromatography is not much helpful. In general, differentiation of species within the *B. fragilis* group is not an easy task, as they demonstrate a great deal of similarity in colony and cell morphology as well as biochemical reactions [14].

7.2.2.2 Concept of PCR-Fingerprinting

Molecular genetic methods, including classic genomic fingerprinting, chromosomal DNA probe hybridization, and species-specific PCR, have been used for identification and characterization of bacterial isolates. For example, new species and changes in nomenclature were increasingly established by using DNA homology studies, especially based on 16S rRNA sequencing and/or 16S-23S rDNA spacer region analysis [15–18]. The latter technique is explained in more detail with *Porphyromonas* (see Sect. 3).

Here we concentrate on PCR fingerprint techniques. These techniques were broadly used for the characterization and identification of bacteria, fungi, and parasites and have proved a versatile method for detection of polymorphisms for identification, characterization and typing of all kinds of micro-organisms. They were described for typing of aerobic and facultative anaerobic bacteria, primarily

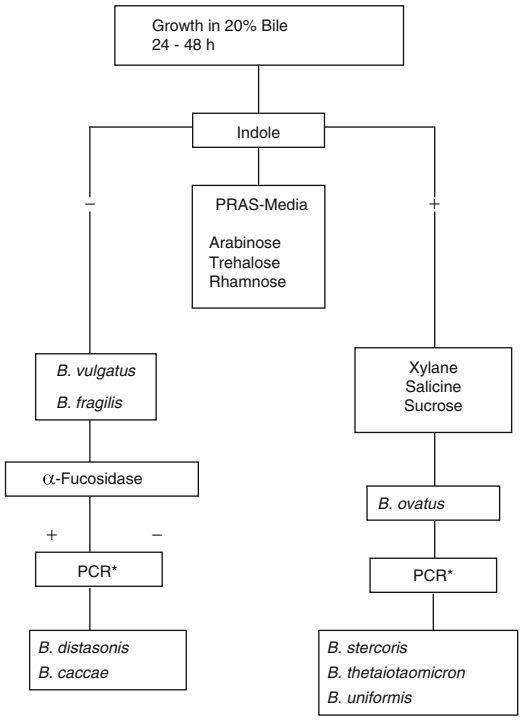

Fig. 7.1 Identification of *Bacteroides* species using molecular and phenotypic methods. *PCR—PCR fingerprinting

with arbitrary primers (AP)-PCR [19–21]. However, completely arbitrary priming lies at one end of a spectrum of possible targeting strategies for fingerprinting. The other end of the spectrum uses primers derived from known near perfect dispersed repeats, for example tDNA-intergenic length polymorphisms. In this spectrum lies a cornucopia of other repeats such as purine-pyrimidine motifs that have been successfully used to produce PCR fingerprints. These mini- and microsatellite repeats are particularly useful because primers directed toward them reveal more polymorphisms between closely related individuals. Primer pairs directed toward rRNA genes are also useful because the rRNA gene clusters evolve more slowly than most of the rest of the genome, which is under less stringent selection pressure. These patterns produced by rDNA directed primers can be used to compare genomes at a higher taxonomic level than is possible with arbitrarily primed PCR [22, 23].

With the use of PCR fingerprint techniques, DNA polymorphisms have been detected that aid in the differentiation of species. Single nonspecific primers or single tDNA primers were used to both identify and characterize selected clinical isolates of *B. fragilis*, *B. thetaiotaomicron* or *B. vulgatus* as well as isolates of *B. distasonis (*reclassified as *Parabacteroides distasonis*) and *B. caccae* with similar biochemical key reactions (Fig. 7.1).

7.2.3 *Bacteroides*: Detailed Protocols

7.2.3.1 *Bacteroides* Strains, Culture Conditions and DNA Extraction

Reference strains were obtained from: American Type Culture Collection (ATCC), US; National Collection of Type Cultures (NCTC), GB; Deutsche Sammlung von Mikroorganismen und Zellen (DSMZ), Germany; and Virginia Polytechnic Institute (VPI), US. In total, 68 indole-negative and 71 indole-positive *Bacteroides* isolates as well as 101 *Bilophila* isolates from blood and wound cultures were obtained from different sites in Germany and in the USA and were subjected to this study. Isolates were grown for 48 h (*Bacteroides* spp.) on Columbia blood agar, or respectively 4–6 days (*Bilophila* spp.) on Bacteroides bile agar in the anaerobe chamber. Two to ten bacterial colonies were subjected into 100 µl sterile distilled water and incubated for 15 min at 95°C. After a short centrifugation step (2 min, 11,000 × *g*) the supernatant was submitted into the PCR mastermix. Alternatively, when pure DNA was needed or inhibitors were present (clinical specimens), extraction was performed with the Qiagen Tissue Kit (Qiagen, Germany) using the instructions from the manufacturer.

7.2.3.2 PCR Amplification and Fingerprinting

Primers: The core sequence of the phage M13 core (5′-AGGTCGCGGGTTCGAATCC-3′) [19]; M13universal (also derived from the phage M13) (5′-TTATGAAACGACGGC CAGT-3′) [20]; the 10mer primer AP3 (5′-TCACGATGCA-3′) [21] as well as the t-DNA-primers T3B (5′-AGGTCGCGGGTT-CGAATCC-3′), T5A (5′-AGTCCGG TGCTCTAACCAACTGAG-3′), and T3A [23] were used as single primers in the experiments (in detail: *Bacteroides* spp.: M13core, M13universal, T3B, T5A and AP3; *B. wadsworthia*: M13core, T3B, T3A). Amplification reactions were performed in 50 µl reaction fluid, which contained 2.5 µl DNA extract, 10× PCR-buffer (10 mM Tris–HCl, pH 8,3; 50 mM KCl; 1,5 mM $MgCl_2$; 3 mM Mg-acetate), 200 µM of each dATP, dCTP, dGTP, and dTTP (Pharmacia Biotech, USA) and 2.5 U Taq DNA-Polymerase (Perkin Elmer Cetus, USA). Negative controls contained PCR approved water instead of DNA. The primers were submitted in a final concentration of 25 pmol or 50 pmol. Samples were amplified as follows: 1 min at 95°C and 1 min at 50°C (universal primers, all other primers) or 30 s at 50°C (tDNA-Primer) or 1 min at 36°C (AP3-Primer), followed by an extension cycle of up to 6 min at 72°C. Reaction tubes were held at 4°C until further analysis. The samples were concentrated to a volume of 20 µl in a vacuum centrifuge (Speed Vac, Savant, USA) and in relation of 1:10 with gel loading solution (Sigma, Germany) was added for gel electrophoresis. All the different PCR assays for an additional group of bacteria were optimized using the Taguchi scheme [24] for the concentration of chemicals and with a temperature gradient the annealing temperature was optimized.

DNA amplicons were separated in submarine electrophoretic apparatuses (Gibco BRL, USA) in 1,2–2,0% agarose gels (depending on the length of the expected

DNA fragments) (Pharmacia Biotech, Germany) in 0.5× TBE-Buffer (Tris–Borate–EDTA, Sigma, Germany). Electrophoretic separation was performed in a 0.5× TBE buffer system gel (5 mm × 25 cm × 20 cm) 5–7 h at 3 V/cm. Amplified products were detected by staining with ethidium bromide (2 μg/ml). Gel images were analyzed by direct visual comparison or scanning the banding patterns (ScanJet IIcx Flatbedscanner; Hewlett Packard, Palo Alto, CA). Absorbance profiles were corrected for gel-to-gel variation on the basis of reference samples run on each gel. Afterwards, the patterns were compared by either calculation of the correlation coefficient between absorbance profiles or by using a band position matching coefficient. Natural groupings of similar patterns were found by clustering the matrix and displaying the results as a dendrogram (GelManager, BioSystematica, Prague, Czech Republic).

For specific gene detection, the amplification of the *bft* gene was performed using the primers and conditions described by Shetab et al. RS-3: TGA AGT TAG TGC CCA GAT GCA GG, RS-4: GCT CAG CGC CCA GTA TAT GAC C, [25] and Kato et al. GBF 101: AGC CGA AGA CGG TGT ATG T , GBF 110: CCC ACT GGC TTC AAA ATC CGA AGC, [8]. For the detection of the *mpII* gene (metalloprotease gene) as well as the BfPAI (*B. fragilis* pathogenicity island) flanking regions the primers and method described by Franco et al. was used (P1T7: GCT GGT AGA CTA CCT GAG TAA GGA GTC, P1T7-1: GCT TCC GTA CCC AGG TAT CTC TCC ATA, P1T3: TTC AAC CTG ATC GAT CCG GAA GAT CCG[*3], P1T3-1: GGT AGT GCT TAT GTC CCT GCA ACC CTA, [26]).

7.2.4 Bacteroides Results

7.2.4.1 PCR Fingerprinting

All strains subjected here were pre-identified using several phenotypic tests (see Sect. 2.2.1). The *Bacteroides* (including *Parabacteroides*) and *Bilophila* strains were screened using primers of different length: M13universal (19mer), M13core (19mer), AP3 (10mer) as well as two different t-DNA primers, T3B (19mer) and T5A (24mer). The primers M13universal, M13 core as well as T3B and T5A produced diverse fragment profiles with species- and strain-specific bands. Nevertheless, amplification products of M13core produced profiles with several main bands. Testing of reference strains of *Bacteroides* species (*B. fragilis* ATCC 25285, *B. thetaiotaomicron* ATCC 29741, *P. distasonis* ATCC 8503, *B. ovatus* DSM 1896, *B. vulgatus* ATCC 8482) showed distinct profiles of all the reference strains. The primer M13core was further used for epidemiological testing but also for species identification and characterization among the strains of one or several *B. fragilis*-group species. About 15 fragments with a length of 0.3–3 kb were determined. For species and group characteristics the primers T3B and T3A were also appropriate. Comparing the profiles of all *Bacteroides* reference strains and phenotypically similar strains such as *Prevotella bivia,* the primer T3B produced about 3–5 main and many more

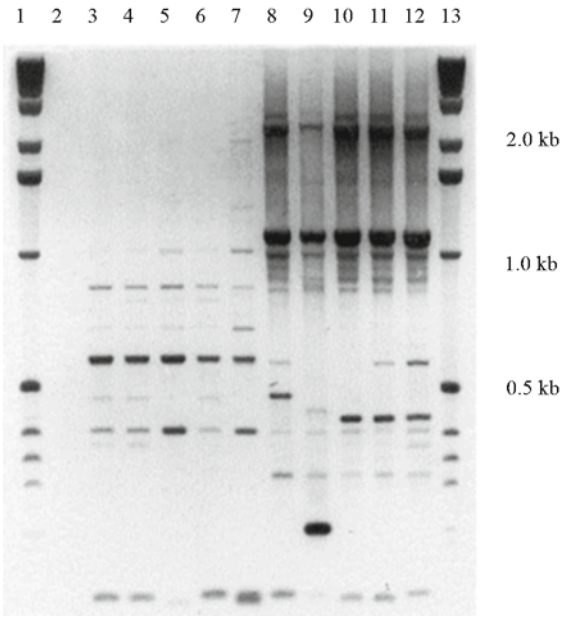

Fig. 7.2 Molecular fingerprinting by using tDNA directed primers (T3B) of *Bacteroides fragilis* strains separating ten strains into two groups (lane 3–7: VPI 2393-like strains; 8–12: ATCC 25285-type-strain-like strains; Lane 1 and 13, marker, lane 2 negative control)

bands with the length of 0.2–4 kb and therefore, seemed to be suitable for species as well as group identification (Fig. 7.2). Welsh and McClelland [23] described the tDNA primers as conserved on the species level, but also determined genus-specific bands in aerobic bacteria. Using these primers in *Bacteroides*, species-specific as well as genus-specific bands were determined. PCR fingerprinting using the T3B primer confirmed the identity of 34 *B. fragilis* isolates. A species-specific fragment with the length of 530 bp could be determined in all the profiles of these strains, showing the potential of typing. The identification of the 11 isolates phenotypically placed into the species *P. distasonis* was also confirmed. Compared to the type strain (ATCC 8503) presenting a main band with a length of 1,480 bp one unusual, indole negative strain was also identified as *P. distasonis*. However, there were a number of discrepancies between the phenotypic and molecular identification of *B. caccae* and *B. vulgatus* isolates. Comparing the species-specific main bands of the type strains of both species, 13 strains were identified as *B. caccae* and 10 strains as *B. vulgatus* (species-specific band with the length of 2.5 kb, data not shown).

7.2.4.2 Characterization of Species and Establishment of Genetic Markers

For *B. fragilis*-typing using the T3B primer, two different unique fingerprint types were established. In total 30 out of the 34 strains showed PCR fingerprint

profiles similar to the type strain ATCC 25285 and established the group I (lanes 8–12 in Fig. 7.2). This group was characterized by 3–4 main bands. A fragment with the length of 1,050 bp was determined as genetic marker for this group. However, 4 of 34 of the *B. fragilis* strains demonstrated similarity with the DNA-homology group II reference strain (VPI 2393), and therefore, were put in the PCR group II (lanes 3 [VPI 2393] to 7 in Fig. 7.2). This group showed many different main bands in comparison to group I. A fragment with the length of 370 bp was determined as genetic marker for this group.

7.2.4.3 Development of Specific PCR for Group resp. Species Detection of *B. fragilis*

For group I the characteristic 1,050 bp fragment and for group II the 370 bp fragment were selectively amplified, cloned, and sequenced. From these group-specific sequences, group-specific primer sequences could be determined, showing the potential of typing methods in designing group/species/strain-specific diagnostic oligonucleotides.

7.2.4.4 Amplification of the Enterotoxin Gene in *B. fragilis* Isolates

Two PCR assays were used to detect ETBF strains. Using several sets of primers (see Sect. 2.3.2), in 10 (11%) clinical isolates the expected 367 bp and 558 bp enhanced virulence genes fragments were amplified. Seven strains (9%) from extra-intestinal infections were ETBF and three blood culture isolates (23%) were ETBF.

7.2.5 Bacteroides Discussion

In preliminary studies, PCR fingerprinting with single primers was demonstrated to reproducibly produce strain-, species-, and group-specific band patterns. Unique band patterns of unknown strains were compared to suitable reference strains and allowed species and subspecies identification. Using fingerprinting with especially primers M13core and T3B, two *B. fragilis* PCR groups were determined, whereas the biochemical groups—because of limitation in appropriate reactions—did not show major differences. Further comparing ATCC 25285 (type strain, reference strain for DNA homology group I [27]) and VPI 2393 (reference strain for DNA homology group II), the separation of two DNA homology groups was confirmed testing clinical isolates from different clinical and geographical sites [27]. The majority of strains belonged to the PCR group I and only a few strains belonged to PCR group II. Performing the amplification reaction with the T3B primer, both groups demonstrated a mixture of specific bands and several group-specific amplicons (Fig. 7.2). This grouping was confirmed using the M13core primer. At the same time this grouping was confirmed by 16S rRNA sequence analysis and it was suggested to establish

the PCR-group II as a second taxon [28]. From our (and the practical, clinical) point of view it is very important to further determine phenotypic differences between the two groups as the biochemical reactions of all the strains so far did not show relevant differences. In contrast, susceptibility patterns of group II strains demonstrated high resistance against betalactam antibiotics, including imipenem (carbapenem) resistance. Appelbaum et al. firstly demonstrated in 1986 changing antibiotic resistance in a few DNA homology group II strains and speculated that this was due to the acquisition of a chromosomally determined metallo-beta lactamase [29]. These results were confirmed using PCR group II strains as well as the resistance testing using the E-test (MICs for imipenem >1 to >32 mg/L). In 1995, the resistance mechanism was described as an endogenous cephalosporinase, encoded by the *cfiA* gene [28]. Referring to the clinical importance and the increasing number of resistant *B. fragilis* isolates, a PCR assay for the differentiation of the PCR group I and II was developed. Group-specific fragments for group I and II were chosen, cloned and sequenced. After sequencing, specific primers for group I and II were developed and their specificity was tested and confirmed in PCR assays. Thus, molecular fingerprinting can be a practical approach and precondition to design clinically relevant diagnostic oligonucleotides (for hybridization and PCR). Furthermore, our molecular fingerprinting studies confirmed the finding of *Bilophila wadsworthia* as a rather homogeneous species, since that, using the M13core primer, common bands were found for all but two of the isolates tested (these two isolates were later found to be preliminarily misidentified and belong to other species, unpublished data by Claros-M). However, using the T3B primer, at least two distinct PCR fingerprint groups were determined. Interestingly, most of the German strains were found in group I (61 of 78 strains, data not shown). Thus, PCR fingerprinting with the T3B primer seems to detect even small epidemiological differences among strains.

7.3 *Porphyromonas*—A Genus Becoming Diverse

The genus *Porphyromonas* currently includes 16 approved species of asaccharolytic, obligate anaerobic, non-spore-forming, Gram-negative, nonmotile, pleomorphic bacilli. Of human origin are five catalase-negative species *P. asaccharolytica*, *P. uenonis* = *P. asaccharolytica*-like [30], *P. endodontalis*, *P. gingivalis,* and *P. somerae* = *P. levii*-like, [31]. Most of the known species are, however, of animal origin, including the catalase-positive *P. canoris*, *P. cangingivalis*, *P. cansulci*, *P. circumdentaria*, *P. gingivicanis*, *P. macacae* (which includes the former *P. salivosa*), and the catalase-negative *P. levii*, *P. crevioricanis* [16], and *P. gulae* =*P. gingivalis-like*, [32]. It has also been shown that "*Oribaculum catoniae*," although saccharolytic, is phylogenetically a member of the genus *Porphyromonas*; thus, it has been reclassified as *P. catoniae* [33]. There are two additional candidates for new species classification, both of which are from humans: PLLO = *P. levii*-like organisms [34–37] and PELO = *P. endodontalis*-like organisms, isolated from extra-oral sites [16, 30, 38] and in 2009 *P. bennoni* was described (for update see http://www.bacterio.cict.fr/). Within the proposed order of *Bacteroidales*, *Tannerella forsythia* (a species related

to *Parabacteroides distasonis* and *P. merdae*, also known as—but grammatically incorrect—*T. forsythensis*), is grouped within the proposed family *Porphyromonadaceae*, so that all three might be close relatives to *Porphyromonas* species [39].

The current study was performed to generate ITS data for most of the type strains of *Porphyromonas* spp. along with *T. forsythia*, *P. distasonis*, and *Prevotella melaninogenica* (outgroups) and to compare a phylogenetic tree deduced from these data with corresponding 16S rRNA gene data. The ITS sequences were further used to clarify the phylogenetic relationship between *P. gingivalis* and *P. gulae*, as well as between—by molecular typing methods—atypical α-fucosidase-negative and—classical—α-fucosidase-positive isolates of *P. asaccharolytica* (which were indeed later on reclassified as *P. uenonis)*. By ITS amplification and sequencing, however, our group published the first hint for this species [15].

7.3.1 Porphyromonas: Methods

7.3.1.1 The General Concept of ITS Determination

Searching for "internal transcribed spacer" in June 2012 reveals 400,000 hits by Google (www.google.com), 840,000 by NCBI-Nucleotide, and about 3,900 by NCBI-PubMed (for the latter see www.ncbi.nlm.nih.gov). Clearly this "Spacer" does attract a lot of interest in research. The reason is that the rRNA internal transcribed spacer (ITS) region is a widely used phylogenetic marker. Ribosomal RNAs are integral parts of the protein synthesis apparatus and thus present in all cellular life forms. On the one hand these molecules and their encoding genes are highly conserved among all prokaryotes (i.e., bacteria and archaea). On the other hand they contain sufficient sequence variability so that evolutionary relationships between different bacteria can be assessed. In addition, with the development of the PCR and sequence technology and recognition of the 16S rRNA gene as outstanding phylogenetic marker gene, specific probes and primers at almost every taxonomic level have been designed and used for detection and phylogenetic characterization of known and novel human pathogens. While the 16S rDNA sequence is a good tool for inferring inter- and intra-generic relationships, the amplification, restriction, and/or sequencing of the 16S–23S rDNA ITS has been suggested to be well suited for typing and identification of bacteria at both the species and the strain level [22], because of marked variation of the ITS in both length and sequence between strains and species (Fig. 7.3). Based on diversities between ITS sequences, it is possible to construct species- and even strain-specific oligonucleotides that can be used to detect or track bacteria in their natural environments including colonized sites in human such as gut, vagina, or the oral cavity. Sequence polymorphism and length variation found in the 16S–23S rDNA ITS are increasingly used as tools for the differentiation of bacterial species and subspecies [40–42]. This is because the higher number of variable sites typical for the ITS sequence [43] can overcome the apparent limitations of the phylogenetic resolution of 16S rDNA in some genera as has been recently described for *Fusobacterium* [40].

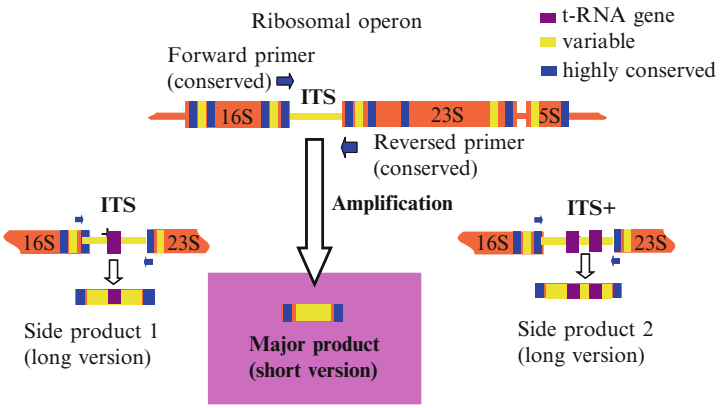

Fig. 7.3 Amplification of the Internal Transcribed Spacer (also known as Ribospacer). Depending on the number of ribosomal operons, the distance between 16S rRNA gene end and 23S rRNA start, and the numbers of t-RNA genes interspersed, the amplicons can be very different by length and sequence.

7.3.1.2 The Selection of Primers

For any PCR, whether quantitative or conventional, the primer selection is not trivial. In this chapter, we discuss this problem based on broad-range primers that bind at highly conserved regions of the 16S rRNA or 23S rRNA genes, both important for constructing an ITS directed PCR (Fig. 7.3). When studying the primary literature the reader will find quite a high number of ITS directed PCR assays. A critical analysis of any article of interest (with respect to the design of the primers, their validation, and the scientific question that the article aims to address) is important. This is, because ITS directed primers never have the potential to encompass the entire spectrum of bacteria and have to be redesigned for any given taxon of interest. This is simply due to the fact that absolute conserved regions with the 16S/23S rRNA gene—although existing—are generally too short to function as primer-binding regions [44]. While several PCR-based pitfalls due to cell lysis techniques or PCR conditions have well been recognized [45, 46], the lack of universality of "universal" PCR primers and its consequences for routine diagnosis are generally not considered. With ever increasing public 16S/23S rRNA gene-databases a reevaluation and possibly redesign of primers is advisable to improve the intended target specificity.

7.3.1.3 The Sample Collection and DNA Extraction

For typing, specimens are taken from pure cultures, but sometimes, e.g., for fast tracking of nosocomial infections, also from the regions associated with infectious disease.

Special care has to be taken in order to avoid contamination during sampling. In the case of abscess puncture or biopsy in the oral cavity or gut the surrounding area

has to be properly isolated and cleaned prior to sampling. Due to the anaerobic life style of all *Bacteroidetes*, rapid transportation into the molecular laboratory is necessary to avoid or limit cell death and subsequent degradation of free DNA. DNA extraction strongly influences the outcome of any PCR reaction. Basic issues are efficient release of bacterial DNA (consider different cell wall properties of Gram-negative and Gram-positive bacteria), co-extraction of PCR-inhibiting substances, accurate storage of DNA extracts (for long-term storage freezing at −70°C is recommended, for short durations storage at 4°C is possible). Frequent freeze-thawing procedures lead to degradation of genomic DNA and should be avoided. If samples have to be used repeatedly aliquots should be made prior to freezing. PCR inhibiting substances co-extracted from human samples can be nucleases (critical in *P. gingivalis* positive oral samples), bile salts, complex polysaccharides in feces, heme, immunoglobulin G, albumin, and lactoferrin in blood [47]. The latter four substances may also be of importance when DNA-samples obtained from bleeding oral sites are analyzed. Besides these substances human DNA itself which is usually co-extracted may interfere with the detection and diagnosis of pathogens [48–50]. Commercial DNA extraction kits are available for numerous applications including DNA extraction from clinical samples, such as tissue or blood. Note that these kits refer generally to extraction of human DNA. Those that were developed for extracting DNA from bacterial cells are based on the evaluation of selected Gram-positive and Gram-negative pure cultures. However, since for oral diagnostics bacterial DNA extraction has to be performed directly from clinical specimen, the best DNA extraction procedure and extraction kit has still to be tested experimentally.

7.3.2 Porphyromonas Detailed Protocol

7.3.2.1 Bacterial Strains, Culture Conditions and DNA Extraction

The following bacterial strains were used: *Porphyromonas asaccharolytica* ATCC 25260^T, RMA 7115 (sacral wound), 7120 (toe), 7178 (endocervix), 8631 (rectal abscess), 9240 (peritoneal), 9603 (abdominal), 9674 (appendiceal fluid), 10263 (peritoneal), 10884, 10898, 10955, 10966, 10997, 11049, 11138, 11258 (the latter eight from pelvic fluid), 11290 (vaginal cupule), 11582 (endometrial pus), 11690 (endometrium), 11666 (endometrial pus), 11805 (pelvic fluid), 12959, 12984, 13273 (the latter three from diabetic foot); *P. cangingivalis* NCTC 12856^T; *P. cansulci* NCTC 12858^T; *P. circumdentaria* NCTC 12469^T; *P. endodontalis* ATCC 35406^T; *P. gingivalis* ATCC 33277^T, RMA 3725 (oral, mandible), 4165 (oral, maxilla), 10371 (peritoneal/abdominal fluid); *P. gulae* ATCC 51700^T; *P. gingivicanis* ATCC 55562^T; *P. levii* ATCC 29147^T; *P. macacae* ATCC 33141, ATCC 49407 ("*P. salivosa*"), as well as *Bacteroides distasonis* ATCC 8503^T, *Tannerella forsythia* ATCC 43037^T, and *Prevotella melaninogenica* ATCC 25845^T. The latter three strains were used for contrast. All strains were cultivated at 37°C on Brucella agar (Anaerobe

Systems, Morgan Hill, Calif.) under anaerobic conditions using an anaerobic chamber. Genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen).

7.3.2.2 PCR Amplification and DNA Sequence Analysis

The 16S primer SPFPorph (5′GTA CAC ACC GCC CGT CAA GCC3′, corresponding to *E. coli* position 1390–1411) as well as the 23S primer SPRPorph (5′TCG CAG CTT ATC ACG TCC TTC 3′, corresponding to *E. coli* position 62 to 42) were designed based on the complete genome of *P. gingivalis* W 83 (GenBank NC 002950); however, the respective regions among bacterial small and large subunit sequences (RDP) are relatively conserved. PCR was carried out using a Biometra Uno I (Biometra) thermocycler in a volume of 100 µl containing 1× PCR buffer, 1.5 mM $MgCl_2$, two units Taq-polymerase, 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim), 10 pmol SPFPorph forward primer, 10 pmol SPRPorph reversed primer, and 100 ng of template nucleic acids. Primer oligonucleotides were synthesized using a DNA synthesizer (OLIGO 1000, Beckman). The amplification was performed using the following temperature profile and 30 cycles: denaturation—1 min at 94°C; annealing—1 min at 52°C; elongation—2.5 min at 72°C. Amplification products (aliquots of 10 µl) were separated electrophoretically on a 2% macro agarose gel in 1× TPE (80 mM Tris-phosphate, 2 mM EDTA, pH 7.5) for a minimum of 18 h at 30 V.

After purification using the Wizard DNA Clean-up system (Promega), the spacer DNA was directly sequenced in duplicate using a Big Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic capillary DNA sequencer (API PRISM 310; Applied Biosystems). Sequences were assembled using the program Vector NTI Suite 9.0 (InforMax) and aligned using the program GeneDoc [51] A phylogenetic tree was constructed by the neighbor-joining method and the programs Clustal W [52], Clustal X [52, 53], and TreeView

7.3.3 Porphyromonas Results

Approximations of ITS lengths were obtained from agarose gels, as demonstrated in Fig. 7.4a. All *Porphyromonas*-reference strains showed a single band between 970 bp (*P. gingivalis* ATCC 33277^T) and 710 bp (*P. circumdentaria* NCTC 12469^T). The four strains of *P. gingivalis* analyzed were almost identical by ITS amplicon length (970–960) and sequence (97–99% similarity, data not shown). In contrast, among 24 clinical isolates of *P. asaccharolytica* and the type strain ATCC 25260^T, the length of the ITS amplicons was more variable and ranged from 1,044 bp (*P. asaccharolytica* RMA 10263, α-fucosidase-negative strain) to 960 bp (*P. asaccharolytica* ATCC 25260^T, α-fucosidase-positive strain) (Fig. 7.4b). In general, it was not possible to differentiate *Porphyromonas* species by comparing ITS

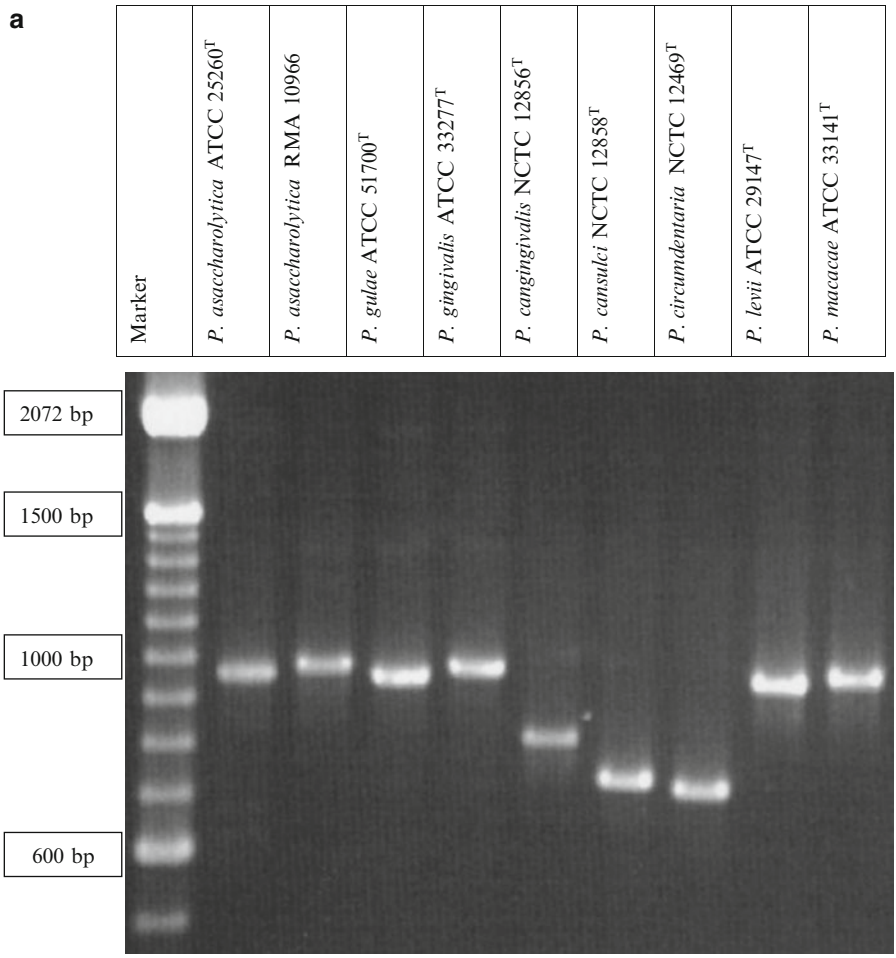

Fig. 7.4 Representative gel-electrophoretic ITS amplification patterns of *Porphyromonas* species to demonstrate inter-species (**a**) and in the case of *P. asaccharolytica* also "intra"-species (**b**) heterogeneity—the latter leading to reclassification of α-fucosidase-negative strains as *P. uenonis*

gel-electrophoretic profiles alone. Further discrimination without need of sequencing might be possible by ITS restriction digest with endonucleases, since we found considerable variation in restriction sites (e.g., *Ava* I, *Apa*LI, *Cla*I, *Eco* RI, *Hin*dIII, *Sma*I). Sequencing the purified ITS amplicons of the *Porphyromonas* strains using SPFPorph and SPRPorph as primers led to nearly ambiguity-free sequence determination by comparing both runs and directions. A database search of tRNA consensus sequences (which should always be performed with ITS) and their comparison with our *Porphyromonas* intragenic spacer DNA revealed no

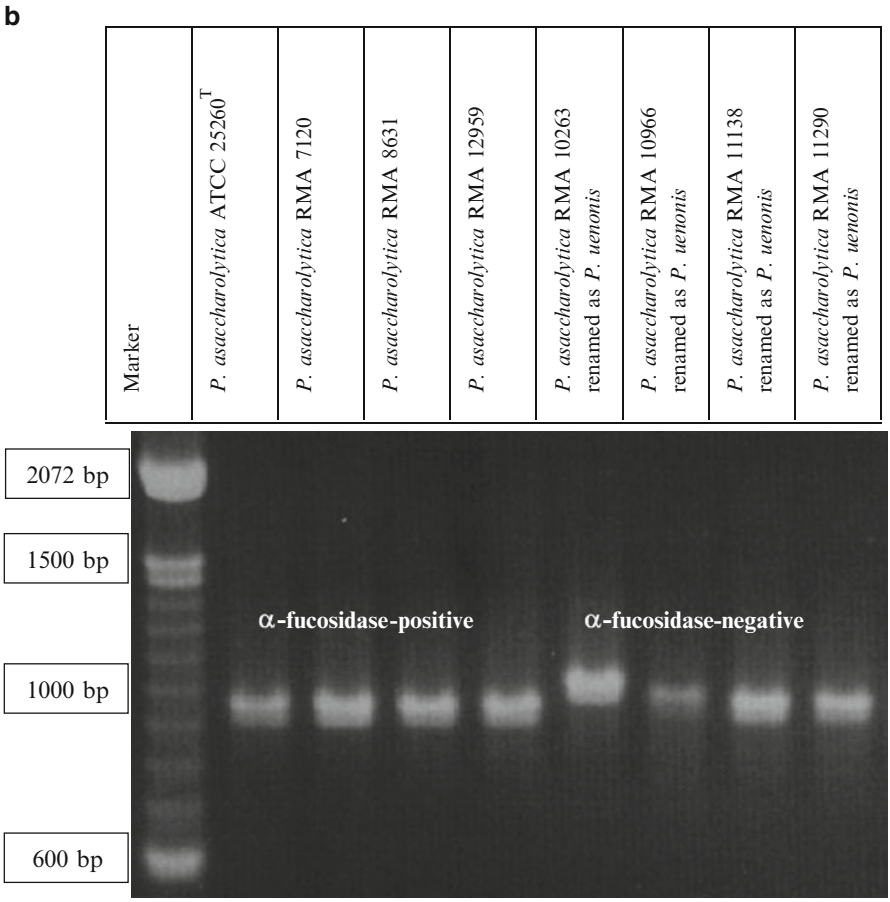

Fig. 7.4 (continued)

matches. Phylogenetic tree reconstruction based on the ITS spacer sequences (short version only in the case of *P. melaninogenica*) is demonstrated in Fig. 7.5. The different strains of *P. gingivalis* matched on a 97–99% level and the two *P. macacae* ATCC strains (ATCC 49407 was formerly referred to as *P. salivosa* and then reclassified) matched on a 94% level; however, *P. asaccharolytica* was more heterogeneous (80–99% range in similarity level). Even more interesting, the latter species, which phenotypically differed in α-fucosidase activity, showed—as expected—two main clusters. Inter-cluster similarity was only 80 to 87%, whereas the intra-cluster similarity was 92–99%.The higher resolution of ITS amplification and sequencing was further used to analyze the relationship between 9 α-fucosidase-positive and 16 α-fucosidase-negative strains of *P. asaccharolytica* and clearly showed that both groups diverged into individual phylogenetic branches [15].

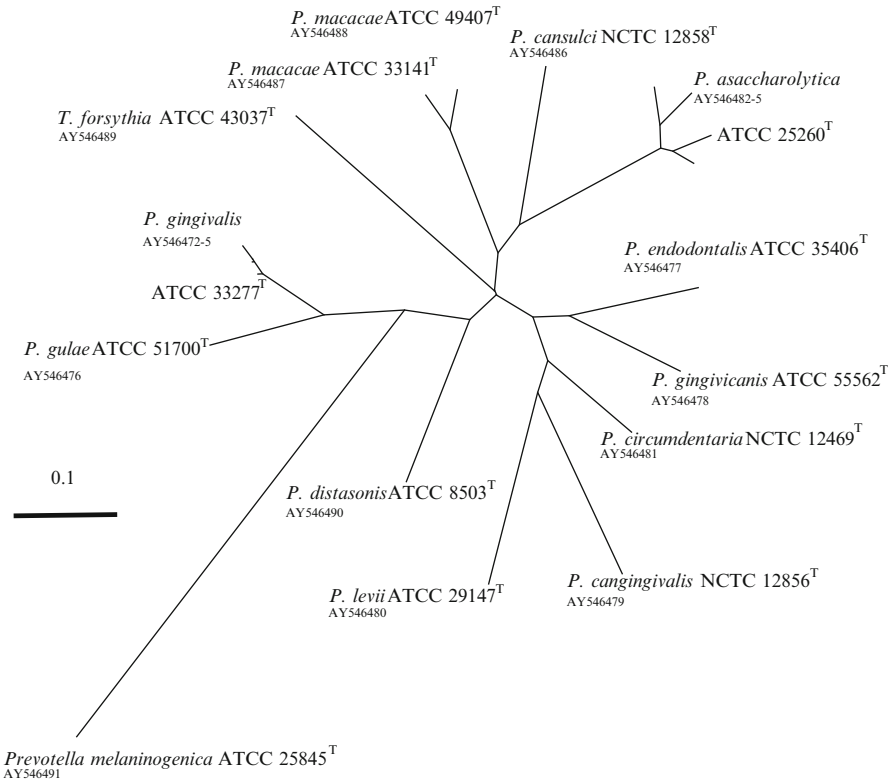

Fig. 7.5 Phylogram (neighbor-joining method) showing the genetic relationships among *Porphyromonas* species based on the DNA sequences of their 16S-23S rDNA spacer regions (GenBank accession numbers are included). (*Para-*)*Bacteroides distasonis* ATCC 8503T, *Tannerella forsythia* ATCC 43037T, and *Prevotella melaninogenica* ATCC 25845T (outgroup, based on the short version of spacer) were included for contrast

7.3.4 Porphyromonas Discussion

PCR amplification of the ITS region using newly designed primers, and subsequent gel electrophoresis of 11 different *Porphyromonas* reference strains plus three clinical isolates of *P. gingivalis* and 24 of *P. asaccharolytica*, showed large heterogeneity in length of amplicons [40]. Furthermore, only one distinct amplification band was produced with *Porphyromonas* species as well as with the relatives *T. forsythia* and *P. distasonis*, unlike for example *Fusobacterium* spp. [40] or many other genera analyzed so far [42, 54, 55], which is mainly due to the number of *rrna*-operons. Within a species, the length of amplicons and the deduced sequence is relatively constant as we have shown for *P. gingivalis* (four strains), *P. macacae* (two strains), and fusobacterial species and subspecies [40]. The high resolution of ITS sequences led to a separation between two clusters of *P. asaccharolytica* strains: one was α-fucosidase-positive

as is typical of the type strain and the other was α-fucosidase-negative. Moreover, eleven of the twelve isolates in the larger α-fucosidase-negative group were isolated from endometrial infection specimens. Thus, the heterogeneity found between the 25 *P. asaccharolytica* strains was a first and later confirmed hint for an unrecognized species, *P. uenonis* [30].

The separation between *P. gingivalis* and *P. gulae* as distinct species was supported by our ITS data; thus, *P. gulae* should not be referred to as the "animal strain of *P. gingivalis*" as it is genetically related but not identical with *P. gingivalis*. Fournier and co-authors, describing *P. gulae*, pointed out the paradox that although this species could be distinguished from *P. gingivalis* phenotypically and by DNA-DNA similarity, the differences between genes encoding 16S rRNA appeared tenuous [32]. They also concluded that the recent divergences of ancestral phyla, e.g., after colonizing different mammalian hosts, could not be sufficiently discerned by 16S information. Again, at least in some genera, ITS data give additional information and enhance phylogenetic resolution if discrepancies between DNA–DNA hybridization and 16S sequencing results are observed.

In conclusion, the ITS spacer region is being used increasingly as an important tool for classification and differentiation of bacterial species. Our study was the first to provide this sequence information for most of the *Porphyromonas* species and their relatives. The higher resolution of ITS helped clarify some of the current problems in molecular taxonomy.

7.4 General Discussion and Final Remarks

For *Bacteroidetes*, especially the clinically relevant *Bacteroides fragilis* and *Porphyromonas* species, PCR based fingerprinting techniques turned out to be ideal for typing since strain/species/group-specific bands can be found (and further used for identification and diagnosis) and only a very small DNA amount is needed. The latter is especially important here, since many obligate anaerobic strains are fastidious or often almost nonviable through oxygen contact and grow very slowly in culture.

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Part III
Oral and Respiratory Pathogens

Chapter 8
Streptococci

Lesley McGee and Bernard Beall

8.1 Introduction

Streptococci are a heterogeneous group of bacteria consisting of more than 50 species. The genus is comprised of a wide variety of both pathogenic and commensal Gram-positive bacteria which are found to inhabit a wide range of hosts, including, but not limited to, humans, horses, pigs, dogs, fish, horses, and cows. Within the host, streptococci often colonize the mucosal surfaces of the mouth, upper respiratory tract, alimentary tract, and genitourinary tract. In certain circumstances, they may also inhabit the skin, heart or muscle tissue. Many streptococci are known to cause human disease, some species being highly virulent and responsible for major diseases. *Streptococcus pyogenes*, *S. pneumoniae*, and *S. agalactiae* are particularly notable as causes of serious infections in man. In recent years, increasing attention has been given to epidemiologic significance of streptococcal species other than the "big three." For example, recently it has been observed in a population-based study that the invasive disease burden attributable to beta-hemolytic *S. dysgalactiae* subsp. *equisimilis* approximated that of *S. pyogenes* [1]. Also, the importance of the viridans streptococcal species is increasingly highlighted in various disease manifestations (dental caries, bacteremia, meningitis, periodontal disease, suppurative infections, pneumonia [2]). *S. anginosus* is also increasingly associated with suppurative infections in children and adults [2]. *S. suis* commonly found as a pathogen in pigs, has become increasingly noted as a cause of severe systemic infections (meningitis and sepsis) in humans [3]. *Streptococcus salivarius* is among the most common normal flora of the mouth and has been increasingly associated with iatrogenic meningitis associated with lumbar puncture [4].

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8.2 Classification of Streptococci

The system for classifying streptococci into major categories was introduced at the beginning of the twentieth century and is based on a series of characteristics of the organisms: (a) colony morphology and hemolytic reactions on blood agar, (b) serologic specificity of the cell wall group-specific substance and other cell wall or capsular antigens, (c) biochemical reactions and resistance to physical and chemical factors, and (d) ecologic features. More recently, molecular genetics have also been used to study the streptococci. Combinations of the above methods have permitted the classification of streptococci for purposes of clinical and epidemiologic convenience, but as knowledge has evolved, new methods have been introduced with the result that several classification systems have been described. In some cases, different species names have been used to describe the same organisms; in other instances, some members of the same species have been included in another species or classified separately. The genera *Enterococcus* and *Lactococcus*, for example, now include some species previously classified as group D and group N streptococci, respectively [5]. In spite of these exceptions to the traditional rules of streptococcal taxonomy, hemolysis and serologic tests can still be used to divide the streptococci into broad categories as a first step in identification and typing of clinical isolates.

8.2.1 Hemolysis on Blood

The type of hemolytic reaction displayed on blood agar has long been used to classify the streptococci. Streptococci are designated as exhibiting either alpha, beta or gamma hemolytic activity. β-hemolysis is associated with complete lysis of red cells surrounding the colony, whereas α-hemolysis is a partial or "green" hemolysis associated with reduction of red cell hemoglobin. Nonhemolytic colonies have been termed γ-hemolytic. Hemolysis is affected by the species and age of red cells, as well as by other properties of the base medium. Group A streptococci (almost always *S. pyogenes*) are nearly always beta-hemolytic; Group B streptococci (*S. agalactiae*) are normally beta-hemolytic but can also manifest alpha or gamma hemolysis. *S. pneumoniae* are normally alpha-hemolytic but can cause β-hemolysis during anaerobic incubation. Most of the oral streptococci are either alpha-hemolytic or nonhemolytic. While the property of hemolysis is not entirely reliable as a streptococcal species marker, it is nonetheless essential for routine rapid screens used for the identification of *S. pyogenes*, *S. pneumoniae* and *S. agalactiae.*

8.2.2 Antigenic Types

The β-hemolytic streptococci are further classified on the basis of a scheme developed by Rebecca Lancefield (1895–1981) that tests the serologic reactivity of carbohydrate antigens (C substance) derived from acid extraction of the bacterial

cell walls [6]. Recognized serogroups are given letter designations from A–H to K–V. Some group antigens are shared by multiple species; however, in general, only a single pathogenic species each comprises groups A (*S. pyogenes*) and B (*S. agalactiae*). Other streptococci with pathogenic potential are found within the β-hemolytic strains of groups C, F and G (*S. equisimilis or S. anginosus*), but only rarely is disease associated with group D (*S. bovis*, *S. durans*, *or S. avium*) or other Lancefield groups. *S. pneumoniae* lacks a group specific antigen and likewise, no group antigen is present in the various viridans streptococcal species (e.g., *S. mutans*, *S. sanguis*, *S. salivarius*, and *S. milleri*). A reliable summary of group antigen associations, biochemical associations, and nomenclature changes within the genus has been published [7].

8.2.3 Molecular Tools

The introduction of DNA-based approaches during the 1960s heralded a new era when genotypic studies combined with chemotaxonomic data allowed major developments to be made in the classification of bacteria in general, and of Gram-positive cocci in particular. One of the most useful tools applied to the revision of the classification system for the *Streptococcus* genus is the application of 16S rRNA gene sequencing [8].

8.2.4 Shared Features in Typing Schemes for the Three Major Streptococcal Pathogens

For the 3 major streptococcal pathogens discussed below, there is a common theme in that for each there is a major surface virulence factor that is expressed as one of many different antigenic types. This is particularly true for pneumococci and GAS that express more than 90 antigenically distinct polysaccharide capsules and more than 100 different M virulence proteins, respectively. In comparison, GBS is known to express a relatively modest array of 9 or 10 different capsular polysaccharides. The pneumococcal capsular polysaccharides have long been utilized in successful multivalent vaccines, while these GAS and GBS surface molecules have been long assessed as vaccine candidates. For each of these three pathogens there has been a long-standing serologic typing scheme of the antigenic types of these major surface virulence factors, and for each there have been DNA-based serotype-deduction methods introduced that suitably substitute for serologic typing. Additionally, for each of these organisms a multilocus sequence typing (MLST) scheme has been developed that determines clonal type. The two approaches (deduction of serologic types and MLST) have revealed that there are strong associations of serological types with given MLST types (STs), and that all three of these species undergo a great deal of intraspecies genetic exchange. For the purposes of epidemiologic typing of large sets of isolates, we have found that deduction of the combination of primary surface antigen serotype and ST serves us best.

MLST is highly discriminating and well suited towards identifying clusters of isolates with identical or closely related genotypes within the three different species. MLST, which employs seven genomically unlinked housekeeping loci [9–11], is highly suitable for the epidemiological analysis of bacterial isolates since it provides results that are portable and available through Internet databases (see www.mlst.net). These databases currently contain more than 6,700 pneumococcal STs, 586 GAS STs, and 551 GBS STs (accessed May 2011). A closely related MLST scheme has been developed for *S. dysgalactiae* subsp. *equisimilis* and *S. canis* which has revealed a significant degree of horizontal exchange of housekeeping loci between *S. pyogenes* and *S. dysgalactiae* subsp *equisimilis* [12, 13].

For all three species, DNA banding pattern based methods have been applied with great success, especially pulsed field gel electrophoresis (PFGE). PFGE and other similar genomic banding pattern assessment methods have proven utility in localized outbreak settings; however, the genotyping information is not readily cross-comparable within global databases, and for this reason is not well suited for large strain distribution studies. In comparison, STs are simple, digital identifiers that can be readily related to component allelic sequence files on the Internet (see www.mlst.net).

8.3 Group A Streptococci (*S. pyogenes*)

Among the pathogenic hemolytic streptococci, *S. pyogenes*, or group A streptococci (GAS), has the most diverse spectrum of acute disease and post-infectious sequelae within the species, with manifestations including acute pharyngitis (“strep throat”), impetigo, rheumatic fever, scarlet fever, glomerulonephritis, streptococcal toxic shock syndrome, and necrotizing fasciitis [14]. GAS express a variety of both cell surface and extracellular virulence factors, with the M-protein being the single most studied and appreciated virulence feature of GAS. The M-typing process can be made more strain-specific by inclusion of two other GAS-characterization methods: T-protein antigen agglutination profiles and sequence typing of the streptococcal serum opacity factor (SOF) [15, 21].

8.3.1 M (emm) Typing

Classic M protein serotyping proved to be invaluable for more than 60 years for resolving more than 100 antigenic types of GAS [16]. A key feature to the M serotyping scheme is that the type-specific, N-terminal region of the protein correspondingly serves as the major type-specific protective antigen. During the past 20 years, unavailability of typing reagents and difficulties in their preparation and maintenance have seen the development of an alternative PCR/DNA-sequencing method called *emm* typing to deduce M serotypes. *emm* typing has greatly extended the utility of the M typing scheme, primarily due to the wide availability of PCR and DNA sequencing technology. In addition to the obvious technical advantages, the

technique offers much less subjectivity than M serotyping in interpretation. For example, GAS strains recovered from relatively remote tropical regions are often nontypeable using M-serotyping; however, strains are always typeable when using M-protein gene (*emm*) sequencing [17]. Classical T agglutination is a useful and relatively simple procedure that is used to augment the strain identification potential of *emm* typing [15], allowing the division of approximately 30 different T protein profiles identifiable using a slide agglutination test [18]. Discovery of the T antigen genes [19] has lead to a straightforward PCR-based scheme proposed to be a reliable replacement [20]. The combination of M (*emm*) type and T agglutination types allows for the quick identification of many global GAS clones [15, 21]. For example, the T1, *emm1* strain is by far the most recognized global GAS strain [22], almost invariably corresponding to MLST type 28 (ST28) [MLST is discussed below]. The same observation holds true for other predominant types such as T3/*emm3* (ST15), T12/*emm12* (ST36), and T28/*emm28* (ST52). Although *emm* type associations with specific GAS clones are strong, there are many known exceptions of *emm* types associated with unrelated clonal types [17, 21, 23].

M protein gene (*emm*) sequence typing is the most widely used method for resolving GAS strains, having replaced the more technically challenging M serologic typing scheme. When examined at a global level, *emm* typing has revealed broad geographic differences in the epidemiology and strain distribution of this species [24]. This genotypic typing scheme is based upon the region of the M protein gene (*emm*) that encodes the type-specific region of the M protein and has become a useful and reliable epidemiologic tool [3, 25, 26]. *emm* typing is independent of *emm* gene expression and can discriminate between biologically distinct isolates that may be only weakly antigenic or nontypeable, allowing for deduction of known M-serotypes and classification of isolates that have new *emm* genes and/or M protein serotypes. This system relies upon the use of two highly conserved primers to amplify a large portion of the *emm* gene [25]. The hypervariable sequence encoding M serospecificity lies adjacent to one of the amplifying primer sequences, allowing for direct sequencing of 150 nucleotides of the 5′ end which displays the highest level of sequence polymorphism; >180 different *emm* types have been described to date [27]. The Centers for Disease Control and Prevention (CDC) maintains a database (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm) that allows for accurate identification of *emm* types using established parameters for identification. The database contains sequences for all identified *emm* types as well as any allelic variations within the defined type-specific region of these types. In addition, this database contains an extensive database of *emm* genes found in *S. dysgalactiae* subsp. *equisimilis* [12, 28] and less commonly in *S. canis* [12].

8.3.2 emm Subtypes

Isolates with small alterations in the *emm* 5′ terminus relative to the major *emm* type subtype can potentially have altered susceptibility to type-specific opsonic antibodies elicited against the M protein [21]; however, such variants are generally effectively

targeted by vaccines raised against the major subtype [29]. The CDC database includes subtypes assigned on the basis of any alterations within the coding region for the predicted 50 N-terminal residues of the processed M protein. A 60 codon region is employed for this subtyping scheme that includes 10 codons of signal sequence that allows precise identification of the mature M protein N-terminus (see http://www.cbs.dtu.dk/services/SignalP/ for reliable program for identifying signal cleavage sites). For example, *emm*68.1 contains a 7 codon deletion within the 5′ 150 bases encoding the mature M protein relative to *emm*68. Subtype information may increase the specificity of epidemiologic information and can be valuable in tracking specific strains. Common *emm* types are subdivided into stable subtypes on the basis of this 150 base type specific region. For example subtype *emm*3 0.1 accounts for the majority of type *emm*3 isolates in the United States (about 75–80%), while subtype *emm*3.4 accounts for about 20% of *emm*3 isolates [30]. A GAS strain with a novel subtype (*emm3.17*) of the very common type *emm*3 was recovered from blood and tissue of an allograft recipient and also from the autopsy of the deceased tissue donor, providing a definitive epidemiologic link [31]. The nomenclature of this subtyping scheme is simple. Any variation within the 180 bases encoding the predicted 50 N-terminal M protein residues plus 10 signal sequence residues relative to the reference strain (designated with a 0.0; e.g., *emm3.0*, *emm6.0*, *emm12.0*, etc.) is assigned a subtype (e.g., *emm*3.1, *emm*3.2, *emm*6.1, *emm*6.2, *emm*12.1, *emm*12.2, etc.). For any *emm* sequence not found to share sequence identity with one of the approximately 1,200 180 base entries in the CDC database (ftp://ftp.cdc.gov/pub/infectious_diseases/biotech/tsemm/), the associated raw data trace file is sent along with relevant strain information to the CDC *emm* database curator for inclusion within the database (ftp://ftp.cdc.gov/pub/infectious_diseases/biotech/tsemm/).

8.3.3 sof Genes from Group A Streptococci

The anti-opacity factor (AOF) type, conferred by the *sof* (serum opacity factor gene) has been widely used as a tool for strain characterization. SOF production occurs in approximately half of all known M/*emm* types, and this production correlates highly with specific M types [32] or *emm* types [21, 25, 33]. The identification of the gene encoding SOF, the *sof* gene, and the subsequent development of tests to detect this gene by PCR amplification and sequencing of a 450–650 bp fragment, have added another tool for GAS characterization [21, 34, 35]. The co-presence of identical *emm* and *sof* sequence types are highly predictive of clonal groups predicted by MLST and can allow rapid resolution in certain instances where different clones have been observed to share the same *emm* type [17]. There is a nearly complete correlation of *sof*-gene presence with SOF production, making the *sof* PCR test a practical alternative to classical phenotypic SOF determination.

8.3.4 *Other Approaches for Typing GAS*

8.3.4.1 *emm* Amplicon Restriction Analysis

To improve sequencing efficiency and economy, restriction digests of *emm* amplicons can be subjected to gel electrophoresis and the resulting enzyme restriction patterns compared. For large collections of isolates, strains with common T-types and opacity factor (OF) reactions, displaying identical *emm* amplicon restriction profiles with *Dde* I and with *Hinc* II + *Hae* III, can then be grouped together, and *emm* sequencing can be performed on a smaller sample (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm). Almost invariably, this *emm* type is highly conserved among the entire group of isolates [36, 37]. *emm* amplicon restriction patterning is a quick method to detect isolate sets that share highly conserved *emm* genes and is particularly useful in outbreak settings. However, it should be noted that certain *emm* types (e.g., types *emm5* and *emm6*) display a large number of different restriction profiles due to the presence of unstable tandem sequence repeats, while other types (for example types *emm1* and *emm12*) display a single predominant restriction profile.

Numerous other useful genotypic methods have been developed for the typing of GAS isolates. These include additional restriction enzyme techniques such as Virtyping [38], ribotyping [39, 40], random amplification of polymorphic DNA (RAPD) [41, 42], and pulsed-field gel electrophoresis (PFGE) [22, 43].

8.4 Group B Streptococci (*S. agalactiae*)

S. agalactiae, or Lancefield's group B streptococcus (GBS), a facultative gram-positive diplococcus with an ultrastructure similar to other Gram-positive cocci, was originally known for causing bovine mastitis and was not demonstrated to be a human pathogen until 1938. Although GBS is generally carried asymptomatically, it can cause invasive disease in newborns, pregnant women, and immunocompromised or chronically ill (e.g., diabetic) adults. Invasive infections in neonates can result in pneumonia, sepsis, or meningitis. Early-onset disease (EOD) occurs within the first week. Late-onset disease (LOD) occurs after the first week and accounts for most meningitis cases and deaths. Because recommendations for intrapartum antibioprophylaxis (IAP) for mothers in labor at risk for GBS infection have been widely implemented in many countries and continue to evolve [44], the incidence of EOD has declined to <1/1,000 births, but the incidence of LOD has remained unchanged [45].

For GBS, serotyping is the most commonly used phenotypic assessment but does not reveal information regarding genetic identity, so many investigators now use genotypic techniques in conjunction with certain phenotypic techniques.

8.4.1 Serotyping

A crucial factor in GBS virulence is the production of an antigenically variable polysaccharide capsule, also used for strain typing. The classification of GBS has evolved over time and currently ten serotypes have been described (Ia, Ib, II-IX) [46]. The distributions of these serotypes, however, vary by geographic location and study population. In the United States serotypes Ia, II, and III, and since the early 1990s serotype V are more commonly associated with invasive disease [47].

Several phenotypic methods have been devised for serotyping GBS and include the Lancefield capillary precipitin method (regarded as the "gold standard") [48], double immunodiffusion [49], coagglutination [50], enzyme immunoassay [51], and latex agglutination [52] methods. Serotyping does, however, have limitations as these tests often have a complicated interpretation, require specific reagents that may not be readily available for routine use in many laboratories and may not be sensitive enough to detect important differences among epidemiologically unrelated strains. In addition, an increasing number of isolates are being classified as nontypeable (~4–9%) by classical serotyping due to mutations in their capsular genes, the presence of reversible nonencapsulated phase variant, or more rarely due to expression of a new capsular serotype [46].

In recent years, various molecular serotyping approaches based on the detection of serotype-specific genes of the capsular region have been developed and have included either PCR in conjunction with sequencing [53], hybridization [54] or enzymatic restriction cleavage pattern analysis [47, 55] and more recently multiplex-PCR approaches [56, 57]. These molecular approaches have made it possible to assign a molecular serotype to many nontypeable isolates and thereby reduce their numbers. Since GBS capsular polysaccharides are candidate components of multivalent vaccines, it is important to deduce capsular serotypes of GBS isolates that have lost the ability to produce capsule subsequent to the infection process. These techniques are also attractive because they are reproducible, specific, and easy to perform and are particularly well adapted for GBS capsular polysaccharide typing in large-scale epidemiological studies [53, 54, 56, 58, 59]. Others studies have additionally used the presence of surface proteins and/or the genes encoding them to characterize the isolates [60]. Although these proteins generally correlate with a capsular serotype, this is not always so.

8.4.2 Protein and DNA Based Fingerprinting of GBS

Population genetic methods have been applied to GBS strains, as well as GAS and pneumococci, to investigate genotypes associated with disease, assess genetic variation within genotypes, and examine the role of recombination in the generation of new genotypes. These include RFLP, PFGE, multilocus enzyme electrophoresis typing (MLEE), and MLST [10, 61–63]. MLST is the more sensitive molecular version of MLEE, a method that plots electrophoretic mobility of housekeeping

enzymes. Based upon housekeeping gene alleles, MLST has subdivided GBS strains into numerous STs [10], with over 500 STs being described to date (http://pubmlst.org/sagalactiae/). Some STs group together into clusters following phylogenetic analyses using eBURST and four major clonal complexes (CCs) (ST1, ST17, ST19, and ST23) have been identified among clinical GBS strains [10]. The distribution of CCs has been shown to differ between colonizing and invasive strains [10, 62, 64]. The ST17 serotype III strains have been associated with neonatal disease in several populations and may have an enhanced ability to cause disease [10, 62, 64, 65].

8.5 *Streptococcus Pneumoniae*

S. pneumoniae (the pneumococcus) is a common colonizer of the respiratory tract and is a prevalent opportunistic pathogen. This organism is a global scourge as a leading cause of bacterial pneumonia, meningitis, otitis media, and sinusitis.

Accurate molecular epidemiologic resolution of pneumococcal isolates is crucial for understanding changes in their population and evolutionary biology as trends in pneumococcal disease are influenced by selective factors in the environment. Serotyping has been the primary method to understand the epidemiology of specific strains of *S.pneumoniae* for decades since vaccines are targeted against the capsular polysaccharides. More recently, additional subtyping techniques and methods for genetic characterization have provided powerful tools for elucidating the epidemiology of pneumococcal disease outbreaks, biologic responses to selection exerted by antimicrobials and vaccines, and the global spread of specific pathogenic clones.

8.5.1 Serotyping

The capsular polysaccharides of *S. pneumoniae* represent a remarkably diverse group of polymers that play an essential role in the virulence of the organism. Serotypes vary in the extent to which they are carried in the nasopharynx and the degree to which they are recovered from different disease states. The Quellung reaction, which uses commercially (or in-house) available factor (typing) sera, is able to divide pneumococci into serogroups and serotypes. These sera have been developed by a process of multiple cross-absorptions, which render them specific for the immunochemical differences between the pneumococcal capsular polysaccharides (CPSs) [66]. At present, 93 individual serotypes are recognized by their patterns of reactivity with the factor sera [67, 68].

Due in part to the large number of serotypes that are encountered in infection and carriage, the standard Quellung reaction test for serotyping pneumococci is labor-intensive and time-consuming, and requires a certain level of experience to be performed satisfactorily. An agglutination method with anti-rabbit IgG-coated latex particles sensitized to pooled and select individual serotype-specific antisera (PCV7

serotypes: 4, 6B, 9V, 14, 18C, 19F, 23F) for serogrouping/serotyping *S. pneumoniae* has been developed and is commercially available [69]. The latex agglutination method is simpler and faster but is intended to narrow the identification down to a group or pool of serotypes and then Quellung can be done using specific antisera for each serotype in the group or pool.

Flow cytometric methods for the serotyping of pneumococci have recently been reported [70, 71]; however, these methods require monoclonal antibodies against the pneumococcal serotype-specific polysaccharide and other reagents that are not readily available to most laboratories.

The high cost of antisera, subjectivity in interpretation, and technical expertise requirements associated with these serologic-based methods have led to a renewed interest in alternative methods to identify the capsular polysaccharides of *S. pneumoniae* and many other bacteria. Central to this renaissance have been the molecular characterization and complete nucleotide sequence determination of the capsular loci for *S. pneumoniae* [72]. One widely used method for PCR serotype deduction involves a sequential multiplex PCR-based serotyping scheme easily adaptable to different serotype distributions [73–75] that presently includes the 40 serotype specificities that are most commonly encountered (http://www.cdc.gov/ncidod/biotech/strep/pcr.htm). This PCR approach has the potential to greatly reduce reliance upon conventional serotyping and provides serotype-determining potential to laboratories that lack type-specific antisera and other reagents needed for conventional serotyping, yet have the equipment necessary for DNA amplification and electrophoresis. This approach has been extended to deducing pneumococcal serotypes present in nasopharyngeal secretions [76, 77] and has great application for deducing serotypes from clinical specimens when causal pneumococcal strains cannot be recovered [78, 79]. It is important to realize that these methodologies and schemes will continue to be refined as additional serotypes are added and primer sets updated to improve specificity. More recently, alternative PCR-based approaches such as real-time PCR and reverse line blot hybridization have also been explored for serotyping pneumococcal isolates [80–82].

8.5.2 Methods for Fingerprinting Pneumococci

Motivated by the emergence of antimicrobial resistance in the 1990s and the spread of resistant organisms worldwide, various subtyping methods have been evaluated to differentiate strains of *S. pneumoniae*. Typing methods such as ribotyping, BOX fingerprinting, ERIC-PCR, pulsed-field gel electrophoresis and restriction fragment-end labeling of small DNA fragments have been widely used to subtype pneumococcal isolates [83] and have long been employed to identify "serotype switch" events where capsular biosynthetic loci have been transferred between distinct genetic lineages [84]. Alterations in penicillin-binding proteins (PBPs) are the major mechanism of resistance to penicillins and cephalosporins in *S. pneumoniae*. The organism possesses several high-molecular-mass PBPs, and most of the high-level penicillin resistance is due to alterations in PBP 1a, 2b, and 2x.

Thus, sequence differences in the genes encoding these PBPs have been exploited to assist in strain-typing *S. pneumoniae*. These methods include restriction fragment length polymorphism (RFLP) analysis of PCR products amplified from *pbp1a*, *pbp2b*, or *pbp2x*, or comparison of sequences of the amplified products [85]. An MLST scheme for *S. pneumoniae* was developed in 1998 [11] using sequence variation within internal fragments (about 500 bp) of seven housekeeping genes. Over 6,000 sequence types have been described at the pneumococcal MLST Web site (http://spneumoniae.mlst.net/). MLST combined with capsular serotyping is an effective means by which to detect serotype switch variants that occur through recombinational gene replacement at the capsular biosynthetic locus. Numerical MLST identifiers have allowed the research community to easily communicate and trace serotype switch events within the pneumococcal population genetic structure [86]; see http://www.sph.emory.edu/PMEN/pmen_criteria.html for nomenclature. MLST allows for insightful analysis of clonal structure and strain emergence within individual successful invasive serotypes. For example, MLST has revealed that invasive serotype 19A disease isolates within the United States are largely comprised of strains that appear likely to have originated within serotypes other than 19A [87], with the most rapidly emerging 19A strains appearing likely to have originated from serotype replacement events within strains formerly expressing 7-valent conjugate vaccine (PCV7) serotypes (4, 6B, 9V, 14, 18C, 19F, 23F).

The use of these typing methods combined with serotyping and antimicrobial-resistance patterns has allowed isolates from different epidemiological regions to be examined for potential relationships and the identification of persistent local and global clones [88]. Data from numerous surveillance projects performed from various countries over the past 30 years show that, although there is considerable diversity among resistant strains, a small number of highly successful clones have emerged within countries and in some cases have achieved massive geographical spread across both national and continental boundaries. A number of these international clones are described by the Pneumococcal Molecular Epidemiology Network that was established in 1997 to standardize laboratory methods and epidemiological definitions for identifying clones of pneumococci (http://www.sph.emory.edu/PMEN). Pneumococci belonging to some of these clones are not only widespread in the geographic sense but also represent a very large proportion of resistant strains in a given epidemiological setting. Isolates belonging to many of these clones have been isolated from pediatric as well as adult disease and as pneumococci colonizing the nasopharynx of healthy children.

8.6 Other Streptococci

Various phenotypic and genotypic approaches have also been developed for determining the molecular epidemiology and population structure of various streptococcal species other than GAS, GBS and *S. pneumoniae*. Recently, a MLST scheme for *S. oralis* [89], an important commensal of the oral microbiota, has been developed to analyze the species and further examine the population structure based on previous

genotypic studies [90]. Other MLST schemes have also been devised to type and speciate viridans and other streptococcal species (http://viridans.emlsa.net/) . Within our laboratory we have very recently found MLST of *S. salivarius* [91, 92] to be useful in tracing the oral carriage source of meningitis associated with lumbar puncture in two different outbreak investigations [93, 94].

S. suis has become an emerging pathogen causing severe systemic infections in humans and the occurrence of outbreaks has increased awareness and improved diagnostics in this species. Of the known 35 serotypes determined by agglutination with a panel of antiserum, only a limited number are responsible for the majority of disease [3]. Serotype 2 is considered to be the most pathogenic and genetic diversity has been studied using various typing techniques including random amplification of polymorphic DNA, PFGE and ribotyping [3, 95]. Analysis of the population structure using MLST [96] has identified 157 distinct STs (http://ssuis.mlst.net/) with ST-1, ST-27 and ST-29 as dominant clonal complexes within this species. In addition, heterogeneity within various zoonotic streptococci such as *S. uberis* and *S. zooepidemicus* has been recently studied using various typing approaches [97, 98].

8.7 eBURST Application to the Molecular Epidemiology of Streptococci

The elegant eBURST algorithm, where strains related to each other through sharing at least 6 of 7 MLST target sequences can be easily connected to create a clonal group, has proved to be a powerful tool for pneumococcal surveillance purposes, allowing for simple resolution of epidemiologically important clonal complexes [99]. In addition, eBURST provides the ability to display likely patterns of diversification of isolates from easily predicted founders within clonal complexes. The simplicity of this tool and its wide potential for streptococcal species in general is enabled by the fact that in streptococci studied to date, genomic changes occur primarily through recombination events between different strains rather than through mutation. Therefore, all MLST targets can be treated equally (regardless of divergence) through eBURST to effectively resolve genetic complexes in streptococcal species (see http://spneumoniae.mlst.net/eburst/, http://spyogenes.mlst.net/eburst/, and http://ssuis.mlst.net/eburst/ where eBURST can be run on entire species databases or selected MLST profiles).

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Chapter 9
Molecular Typing of *Streptococcus mutans*

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9.1 Properties of *Streptococcus mutans*

Streptococcus mutans, a Gram-positive facultative anaerobic bacterium, is generally known to be a pathogen of dental caries and also considered to be one of the oral streptococcal species that can cause infective endocarditis since it was reported to be recovered from 8 to 10% of patients with endocardial disease [1] (Fig. 9.1). *S. mutans* is one of the members of the "mutans streptococci" group, which also consists of *Streptococcus sobrinus*, *Streptococcus cricetus*, *Streptococcus rattus*, *Streptococcus ferus*, *Streptococcus macacae*, and *Streptococcus downei*. Mutans streptococci were previously classified into eight serotypes based on the chemical composition of their serotype-specific polysaccharides, among which five serotypes (*a* through *e*) were designated in 1970, followed by three additional serotypes (*f*, *g*, *h*) determined during the next decade. *S. mutans* (*c*/*e*/*f*) and *S. sobrinus* (*d*/*g*) are detected in humans, while *S. cricetus* (*a*) and *S. rattus* (*b*) strains are mainly identified in hamsters and rats, respectively. In addition, *S. ferus* (*c*) was reported to be isolated from rats, and *S. macacae* (*c*) and *S. downei* (*h*) were isolated from monkeys. Among the mutans streptococci, *S. mutans* is the most frequently identified in the

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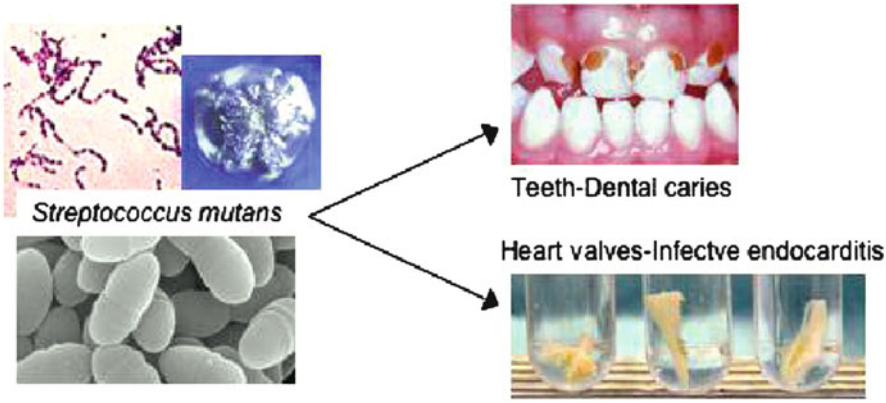

Fig. 9.1 *Streptococcus mutans* and its associated diseases

human oral cavity, followed by *S. sobrinus*. On the other hand, the presence of the mutans streptococci in the oral cavities of animals is considered to result from the ingestion of sucrose contained in feed.

The serotype-specific polysaccharides of *S. mutans* are known to be composed of rhamnose-glucose polymers, with a backbone of rhamnose and side chains of glucose polymers [2]. The chemical linkage of each rhamnose unit is common in each *c*/*e*/*f* serotype (α-1,2 and α-1,3 repeatedly), while that for glucose side chains is different; α-1,2 for serotype *c*, β-1,2 for serotype *e*, and α-1,3 for serotype *f*. The distribution frequency of serotype *c* strains in the oral cavity is the highest with a rate of 70–80%, followed by serotype *e* (approximately 20%) and *f* (less than 5%). Strains, which could not be classified into any of *c*/*e*/*f* serotypes, have been described in the literature from subjects in several countries. However, details of the chemical composition of their serotype-specific polysaccharides have not been investigated. In 2004, the 9th serotype "*k*" was designated for the non-*c*/*e*/*f* serotype *S. mutans* strains which were isolated from the blood of the patients with bacteremia after tooth extraction and infective endocarditis complicated with subarachnoid hemorrhage [3].

Analysis of the distribution of serotype *k* strains revealed that the detection frequency in the oral cavity of Japanese children was 2–5%, which was shown to be consistent with that of Thai subjects [4]. In addition, several *S. mutans* strains of non-*c*/*e*/*f* serotypes isolated in Finland and UK were classified into serotype *k* [5, 6]. There have been several reports demonstrating the presence of non-*c*/*e*/*f* strains but an estimation of the presence of serotype *k* strains is not possible due to the lack of adequate description of the chemical composition of the serotype-specific rhamnose glucose polymers. On the other hand, a recent study in Chile considered the possibility of the presence of serotype *k* for the strains of non-*c*/*e*/*f S. mutans* serotypes isolated [7]. Taken together, these studies suggest that the serotype *k* strains are prevalent worldwide.

In addition to polysaccharide antigens, cell surface protein antigens are important for the virulence of *S. mutans* in dental caries. Among the various cell surface

antigens, an approximately 190-kDa protein antigen (PA), glucosyltransferases (GTFs), and glucan-binding proteins (Gbps) are known to be major virulence factors for *S. mutans* [8–10]. PA, also referred to by other names (PAc, SpaP, antigen I/II, antigen B, SR, IF, P1, and MSL-1) is correlated with the sucrose-independent initial adhesion to tooth surfaces by the bacterium. In addition, GTFs are composed of three types (GTFB/GTFC/GTFD), and are known to be associated with sucrose-dependent adhesion. GTFB and GTFC, located on the cell surface, mainly synthesize water-insoluble glucans, which contain a high degree of branching of α-1,3-glucosidic linkages, whereas GTFD, released into the culture supernatant, produces water-soluble glucans that are predominantly linear polymers linked by α-1,6-glucosidic bonds, similar to dextran.

The complete genome of *S. mutans* strain UA159 (serotype *c*) was sequenced in 2002 by a team at the University of Oklahoma Health Sciences Center, which revealed that it was composed of 2,030,936 bp and contained 1,963 ORFs [11]. Detailed information is available in the Oral Pathogen Sequence Database provided by the Database Team at the Bioscience Division of Los Alamos National Laboratory (http://www.oralgen.lanl.gov/). Recently, the complete genome of another *S. mutans* strain NN2025 (serotype *c*) isolated from a Japanese child with severe dental caries was sequenced, which showed that it was composed of 2,013,587 bp and contained 1,869 ORFs [12]. When comparing the complete genomes of the two strains, core-genome was shown to be highly conserved, whereas a large genomic inversion between homologous ribosomal operons across the replication axis was identified. In addition, at least 25 different regions, which might be transferred following conjugation transfer or mediated by insertion elements, were identified in the two strains. At this moment, the complete genome of an additional *S. mutans* strain LJ23 (serotype *k*) is now being sequenced in order to identify the serotype-*k* specific genomic features by comparison with UA159 and NN2025.

9.2 Detection of *S. mutans*

In the early 1990s, DNA probe methods targeting *gtf*s and other genes were constructed to detect *S. mutans*. However, these are hampered by complex procedures and low sensitivity and one of the studies demonstrated that more than 300 pg of DNA and as many as 2×10^5 cells would be required for detection [13]. In the middle of the 1990s, the PCR-based approach for *S. mutans* detection was introduced, which was regarded as rapid, sensitive and relatively simple method. The *spaP* gene encoding the 190 kDa-protein antigen (PA) [14] and the *dexA* gene encoding extracellular dextranase were targeted for construction of species-specific sets of primers [15] (Table 9.1). The sensitivity was drastically increased with a lower limit of 1 pg of chromosomal DNA or 12 colony-forming units of *S. mutans* cells. Subsequently, several molecular methods for detecting *S. mutans* DNA in specimens from dental plaque and saliva have been reported. In addition, cardiovascular specimens, such as those from heart valves and atheromatous plaques, have been examined [16, 17].

Table 9.1 PCR primers for identification of *S. mutans*

Target gene	Name	Sequence (5′ to 3′)	Product Size (bp)	References
spaP	Sense	AAC GAC CGC TCT TCA GCA GAT ACC	192	[14]
	Antisense	AGA AAG AAC ATC TCT AAT TTC TTG		
dexA	SD1	TAT GCT GCT ATT GGA GGT TC	1,272	[15]
	SD2	AAG GTT GAG CAA TTG AAT CG		

Table 9.2 PCR primers for identification of *S. mutans*

Target gene	Name	Sequence (5′ to 3′)	Product Size (bp)	References
gtfB	GTFB-I	ACT ACA CTT TCG GGT GGC TTG G	517	[20]
	GTFB-R	CAG TAT AAG CGC CAG TTT CAT C		
gtfD	MKD-F	GGC ACC ACA ACA TTG GGA AGC TCA GTT	433	[21]
	MKD-R	GGA ATG GCC GCT AAG TCA ACA GGA T		
16S rRNA	8UA	AGA GTT TGA TCC TGG CTC AG	1,505	[24]
	1492R	TAC GGG TAC CTT GTT ACG ACT T		
16S rRNA	Forward	GGT CAG GAA AGT CTG GAG TAA AAG GCT A	282	[25]
	Reverse	GCG TTA GCT CCG GCA CTA AGC C		
gorESL	ES.5-29	TAA AAC TAG GFG AHC GWR TBG T	430	[29]
	EL.35-18R	CKK GCA TCT GCT GAA AAT		
ddlA	Forward	ATT GAA GGC GAG CCT TTA GAA AG	351	[30]
	Reverse	GTT GCT ATT GTC CTA G		
sodA	*d1*	CCI TAY ICI TAY GAY GCI YTI GAR CC	480	[31]
	d2	ARR TAR TAI GCR TGY TCC CAI ACR TC		

The lower limit of detection for *S. mutans* DNA was reported to be detected in heart valve specimens extirpated from infective endocarditis patients, in which multiple species were identified in each specimen [18]. Thus, it is possible to speculate that *S. mutans* is one of the possible etiological agents and/or it is incidentally detected during transient bacteremia. As for atheromatous plaque, the high detection rate of *S. mutans* DNA does not necessarily mean a direct association of *S. mutans* with atheromatous plaque formation [19]. It is advantageous that PCR methods are very sensitive, however, careful interpretation of the results is required since bacterial DNA from nonviable and/or incidentally disseminated strains can be identified as positive reactions.

Table 9.2 summarizes the commonly used primer sets for *S. mutans* detection, among which those designed based on the *gtf* genes are widely used. Many oral streptococcal species reside in dental plaque, and the glucan synthesizing by the glucosyltransferases encoded by the *gtf* genes is one of the major factors in dental plaque formation [9]. The species-specific sets of primers have been constructed based on the differences in the nucleotide alignments of the *gtf* genes among several oral streptococci. The primer sets for *S. mutans* designed based on the *gtfB* or the

gtfD genes are widely used [20, 21]. As for the methods using the *gtfD* sequence, the lower limit of detection for *S. mutans* DNA was reported to be 1.5 pg, indicating that this method is very sensitive. In addition, the methods for quantifying the numbers of *S. mutans* cells were developed using real-time PCR with the primer set SmF5 (5′-AGC CAT GCG CAA TCA ACA GGT T-3′) and SmR4 (5′-CGC AAC GCG AAC ATC TTG ATC AG-3′) targeting the *gtfB* gene [22]. It was reported that high levels of *S. mutans* in the parents is one of the important factors for vertical transmission into children [23]. Thus, this method could be one of the possible tools for identifying subjects with high risk for transmission. In addition, it could also be used to determine the number of the bacterial cells in cardiovascular specimens, which might lead to the identification of specific pathogenic bacterial species when multiple species are identified in each specimen by PCR.

The other molecular approach for detecting *S. mutans* is the restriction fragment length polymorphism (RFLP) of amplified 16S rRNA fragments, in which approximately 1,500 bp fragments are amplified with the universal primers 8UA and 1492R followed by digestion with *Hpa*II [24]. In addition, another primer set based on 16S rRNA alignments [25] has been modified to amplify approximately 1,500 bp lengths of 16S rRNA followed by nested PCR amplification of an internal 282 bp region [24]. However, it was reported that false positive could result since the 16S rRNA sequence of the mutans streptococci and neighboring group are quite similar [26]. On the other hand, the determination of the entire 16S rRNA sequence amplified by the primer sets 8UA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1540R (5′-AAG GAG GTG ATC CAG CC-3′) was used to confirm that the tested strain was truly a *S. mutans* [27]. This obviates misleading results although it is more time consuming and expensive than PCR methods with species-specific primer sets.

The *groESL* genes encoding 10-kDa (GroES) and 60-kDa (GroEL) heat shock proteins are reported to be ubiquitous and evolutionary highly conserved genes [28]. However, the *groESL* sequence is known to be less conserved as compared to the 16S rRNA sequence, indicating the possibility for its application in the differentiation of species with high degrees of similarity in their 16S rRNA sequences. The primer set for identification of mutans streptococci using the *groESL* genes was designed to amplify the fragment containing a region of partial *groES*, partial *groEL*, and the *groES-EL* spacer [29]. Since the nucleotide length of the *groES-groEL* spacer is varied among each species (111 bp for *S. mutans*, 218 bp for *S. sobrinus*, 200 bp for *S. cricetus*, 125 bp for *S. rattus*, and 310 bp for *S. downei*), the molecular sizes of the positive bands are different depending on the species. The other species-specific sets of primers were also constructed based on the nucleotide alignment of the *ddlA* gene encoding D-alanine:D-alanine ligase, which is known to be essential for bacterial cell wall synthesis [30]. In addition, a method for sequencing the internal fragment representing 85% of the *sodA* gene encoding manganese-dependent superoxide dismutase, was also developed, which discriminates between a large numbers of the various streptococcal strains [31].

The 16S–23S ribosomal RNA intergenic spacer (ITS) region, known to contain low levels of intraspecies variation and high levels of interspecies divergence, can also be used for speciation of *S. mutans*. PCR using universal primers 13BF (5′-GTG

Table 9.3 Primers used for broad-range PCR

Target gene	Name	Sequence (5′ to 3′)	References
16S rRNA	536f	CAG CAG CCG CGG TAA TAC	[33]
	1050r	CAC GAG CTG ACG ACA	
16S rRNA	PA	AGA GTT TGA TCC TGG CTC AG	[34]
	PD	GTA TTA CCG CGG CTG CTG	

AAT ACG TTC CCG GGC CT-3′) and 6R (5′-GGG TTY CRT TCR GAA AT-3′) was designed based on the sequence of the 3′-region of the 16S rRNA gene and the 5′-portion of the 23S rRNA gene [32]. This amplifies fragments of variable sizes depending on the species. When the specimens contain *S. mutans* DNA, the amplified fragments include the 387 bp or 388 bp regions of ITS. Determination of the nucleotide alignment is initially performed, after which the identification of *S. mutans* is made by comparing the sequence of species-specific ITS and that of the specimens.

It is possible that the conventional methods for identification of bacterial species fail to identify phenotypically aberrant strains. On the other hand, the broad-range PCR and sequencing method, in which full and partial 16S rRNA nucleotide alignments are determined, is a reliable tool. As compared to PCR with species-specific sets of primers, the broad-range PCR and sequencing method enables the identification of multiple species in the specimens. Several primers for broad-range PCR methods have been developed and the amplified fragments are then sequenced, for comparison with those in the GenBank, EMBL, and DDBJ databases using the gapped BLASTN 2.0.5 program obtained from the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST/) [33, 34] (Table 9.3). Identification to the species level is generally defined as a 16S rRNA sequence similarity of more than 99% with that of the prototype strain sequence in the databases. This approach is widely used for the investigation of bacterial profiles in the clinical specimens such as saliva and dental plaque as well as with cardiovascular specimens.

S. mutans strains are easily isolated from oral specimens, such as saliva and dental plaque, using selective medium and Mitis-salivarius agar plates containing bacitracin (MSB plates) [35]. On the other hand, *S. mutans* is occasionally isolated from the blood of the patients with bacteremia and infective endocarditis. However, it is difficult to isolate the strains from blood specimens once antibiotic treatments are initiated. The molecular methods enable detection of *S. mutans* using bacterial DNA extracted from even nonviable cells, which should be regarded as the most advantageous aspects in the use of molecular approaches.

9.3 Differentiation of *S. mutans* and *S. sobrinus*

S. mutans is highly prevalent in the oral cavity of humans, with a detection rate ranging from 74 to 94%, while *S. sobrinus* is known to be less prevalent [36]. In general, *S. mutans* is reported to be associated with coronal caries, whereas *S. sobrinus* is considered to be correlated to the lesions found on the smooth surfaces [36, 37].

It is generally known that subjects harboring both *S. mutans* and *S. sobrinus* have a significantly higher caries experience than those with only *S. mutans* [38, 39].

S. mutans and *S. sobrinus* are easily discriminated based on the rough and smooth colonies, respectively, on Mitis-salivarius agar plates. However, the GTF-defective strains of *S. mutans* are known to show smooth colony morphology on the agar although the distribution frequency is extremely low [40, 41]. Thus, it is possible that phenotypic variation prevents the appropriate discriminations of *S. mutans* and *S. sobrinus*. However, PCR-based approaches using species-specific primers for each species are not influenced by the phenotypic variations of *S. mutans*. The primer sets for *S. mutans* were designed based on the *gtfB* or *gtfD* genes of *S. mutans*, whereas *gtfI* was used for construction of the primer sets for *S. sobrinus* [20, 21].

One of the other molecular approaches is the PCR-RFLP method. In this method, the 4-kb region of the ribosomal RNA (*rrn*) operon, which most bacteria possess several copies for, with a high degree of homology, is amplified by PCR, followed by the comparison of the digestion patterns of the amplified fragments following *Hin*fI, *Mbo*I, or *Taq*I digestion [42]. Another approach is the chromosomal DNA fingerprint (CDF) and arbitrarily primed (AP)-PCR methods [43]. The CDF method can discriminate between two major patterns after *Hae*III digestion of the chromosomal DNA of each strain. One is designated as the CDF-1 group with restriction fragments equal to or greater than 6.6 kb in size and the other the CDF-2 group with fragments less than 6.6 kb. All of the tested *S. mutans* strains are classified as CDF-1, whereas most of the *S. sobrinus* strains were classified as CDF-2.

As for AP-PCR approach characterized by the short length of primers and low annealing temperatures, the primer OPA-02 (5′-TGC CGA GCT G-3′) is the most commonly used for the analysis of *S. mutans*, followed by OPA-13 (5′-CAG CAC CCA C-3′). This choice is primarily due to the appearance of readily identifiable electrophoretic products with the former compared to the other 40 sets of the primers [44] (Fig. 9.2). *S. mutans* and *S. sobrinus* strains showed similar patterns in each group, which are reported to consist of the major common amplified fragments of 782 bp and 1,070 bp, respectively. In addition, OPA-03 (5′-AGT CAG CCA C-3′), OPA-05 (5′-AGG GGT CTT G-3′) and OPA-18 (5′-AGG TGA CCG T-3′) were also used in several studies [44].

9.4 Classification of Serotypes

The genes involved in the biosynthesis of serotype-specific polysaccharides are estimated to be located in four different regions and those in strain UA159 (serotype *c*) are illustrated in Fig. 9.3. There are multiple enzymes required for the biosynthesis of the polysaccharides and the biochemical steps and their relevant genes have been identified [1]. The enzyme RgpG encoded by *rgpG* is proposed to be involved in the first step in the formation of the polysaccharides catalyzing the transfer of *N*-acetylglucosamine-1-phosphate to a lipid carrier [45]. On the other hand, the

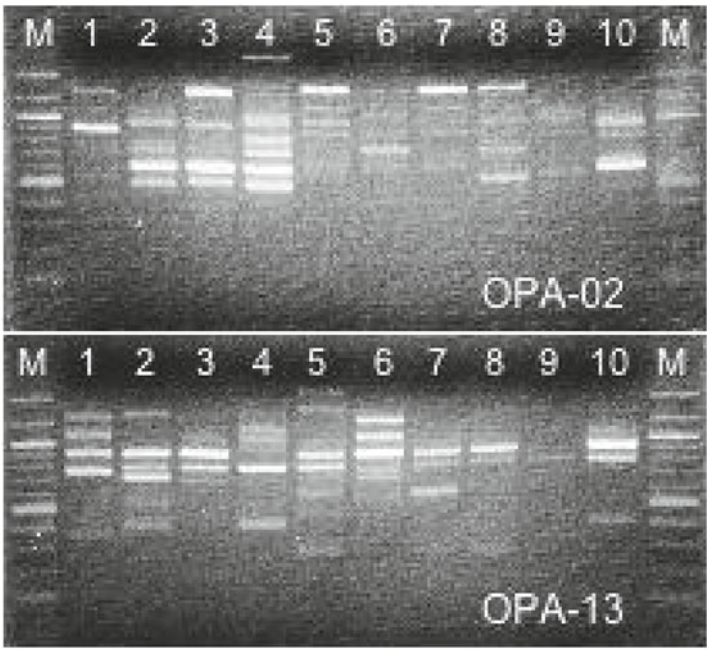

Fig. 9.2 Electrophoretic appearance of the results of arbitrarily primed (AP)-PCR for ten clinical strains (*Lane 1–10*) using OPA-02 and OPA-13 primer sets

basic units of the rhamnose polymers and glucose side chains are considered to be dTDP-L-rhamnose and UDP-D-glucose, respectively. These units are synthesized from the UDP-D-glucose-1-phosphate by the actions of multiple enzymes encoded by their respective genes [46, 47]. The *rmlA*, *rmlB*, *rmlC*, and *rmlD* genes are known to encode the enzymes that catalyze the pathway from UDP-D-glucose-1-phosphate to the rhamnose units [46, 48], and the *gluA* gene encoding GluA is known to be involved in the biosynthesis of the units of the glucose side chain [49].

The *rgpA*, *rgpB*, and *rgpF* genes encoding RgpA, RgpB, and RgpF, respectively, are reported to function in the polymerization of the rhamnose units [47]. Specifically, RgpA is proposed to be associated with the first rhamnose unit, whereas RgpB and RgpF are presumed to be correlated with the polymerization of the even and odd numbers of rhamnose units from the second unit, respectively [50]. In addition, the *rgpE* gene encoding RgpE is considered to be involved in the side-chain formation by glucose units [47]. Furthermore, RgpH was shown to encode a glucosyltransferase, while RgpI is thought to control the frequency of branching [51]. As for polysaccharide export, the *rgpC* and *rgpD* genes encoding RgpC and RgpD were demonstrated to regulate this function [47].

As compared to serotype *c/e/f* strains, the region from downstream of *rgpF* to the upstream of ORF12 was demonstrated to be highly variable among each serotype [38]. Using the differences in the nucleotide alignments in these regions, primer sets

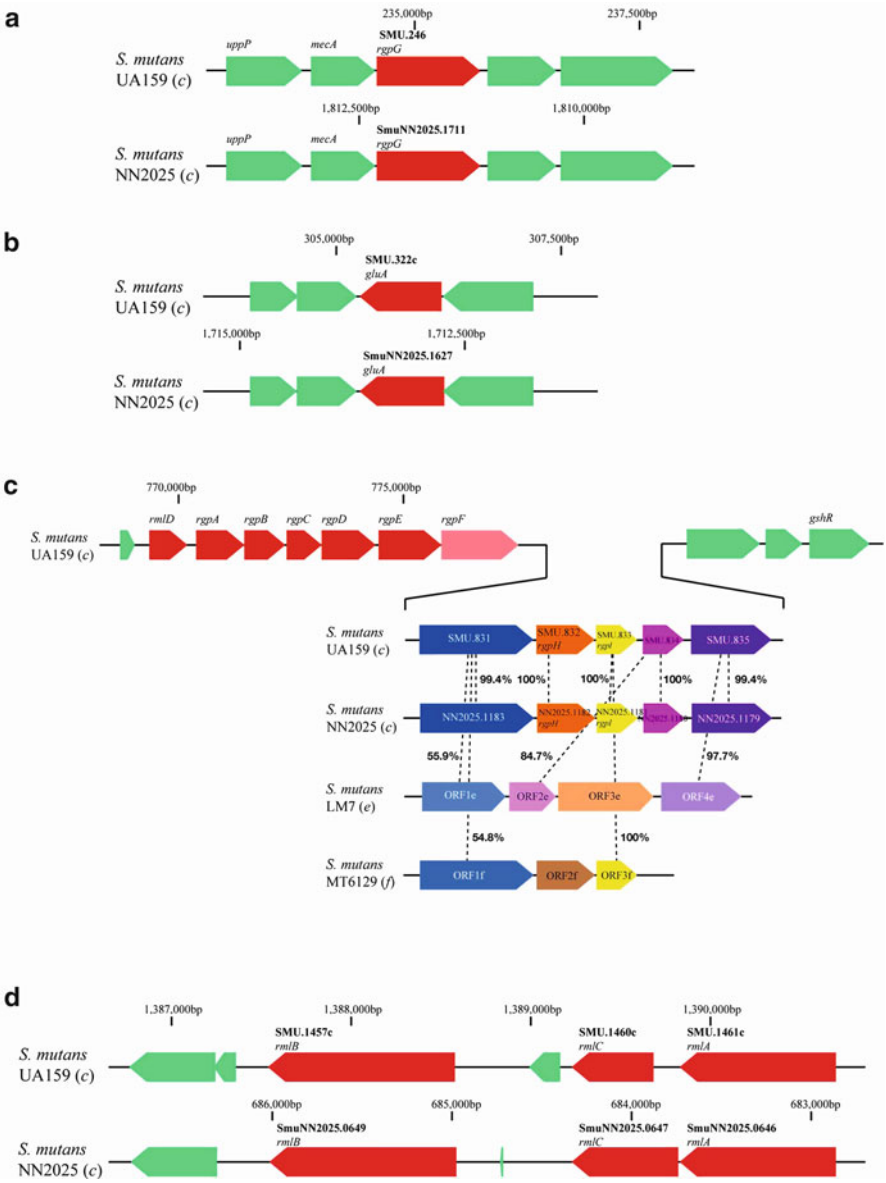

Fig. 9.3 Genes involved in the biosynthesis of the serotype-specific polysaccharide of *S. mutans*. The genes were located four different regions (**a**–**d**). *rgpG* (**a**) and *gluA* (**b**) genes are completely conserved between two sequenced serotype *c* strains (UA159 and NN2025). However, genes located downstream of *rgpF* are quite diverse (**c**). *rmlA-C* genes are also conserved between the two serotype *c* strains, but several differences are found in the intergenetic regions (**d**)

Table 9.4 PCR primers for determination of serotypes

Serotype	Name	Sequence (5′ to 3′)	Product size (bp)	References
c	SC-F	CGG AGT GCT TTT TAC AAG TGC TGG	727	[38]
	SC-R	AAC CAC GGC CAG CAA ACC CTT TAT		
e	SE-F	CCT GCT TTT CAA GTA CCT TTC GCC	517	[38]
	SE-R	CTG CTT GCC AAG CCC TAC TAG AAA		
f	SF-F	CCC ACA ATT GGC TTC AAG AGG AGA	316	[38]
	SF-R	TGC GAA ACC ATA AGC ATA GCG AGG		
k	CEFK-F	ATT CCC GCC GTT GGA CCA TTC C	296	[52]
	K-R	CCA ATG TGA TTC ATC CCA TCA C		

for identification of each *c*/*e*/*f* serotype were constructed. On the other hand, no drastic differences in the nucleotide alignments between serotype *c* and *k* strains were identified in that region [52]. However, a serotype *k*-specific alignment is present in the 5′ one-third end of the *rgpF* gene, upon which the serotype-*k* specific set of primers was constructed. It should be noted that these alterations in the nucleotide alignments in this region for the serotype *k* strains are considered to be inconsequential for the observed variety of glucose side chains in the serotype-specific polysaccharides [53]. Table 9.4 lists the primers for determination of each serotype *c*/*e*/*f*/*k* strain of *S. mutans* [38, 52]. PCR detection system using these primer sets were demonstrated to be very sensitive, with the minimum number of cells detected being 5–50 per reaction.

9.5 Identification of Virulent Strains

Considering the prevention of dental caries, the approaches used should be ideally based on the common risk factors for dental caries [54]. Thus, the identification of subjects with highly virulent strains could be beneficial for the prevention of dental caries. This should be true also when considering the pathogenesis of *S. mutans* in blood. It is generally accepted that considerable phenotypic variations exist within *S. mutans* species, which is derived from a consequence of a variety of genetic events, such as point mutations, translocations and inversions [35]. Therefore, some of the strains show strong virulence and others are regarded as weak virulent strains. In order to develop molecular methods to identify subjects who harbor the highly virulent *S. mutans* strains, several PCR approaches using the extracted bacterial DNA from the specimens have been evaluated.

One of the methods for possible clinical use in estimating the risk of dental caries in subjects is the identification of the multiple serotypes of *S. mutans* in specimens from the oral cavity by PCR with serotype-specific sets of primers [38]. The use of such methods is supported by the evidence that dental caries scores for preschool children with multiple serotypes of *S. mutans* were shown to be significantly higher than those with a single serotype or with no detectable *S. mutans*. It should be noted

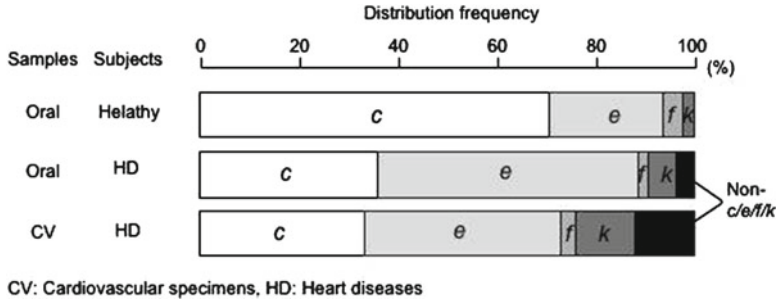

Fig. 9.4 Serotype distribution of oral or cardiovascular specimens from healthy subjects or patients with cardiovascular diseases

that the risk for subjects should be estimated based upon their clinical conditions, such as the number of lesions or fillings, in addition to the results of the molecular analyses. The other study showed the unique distribution of the serotypes in the oral and cardiovascular specimens in subjects with cardiovascular diseases (Fig. 9.4) [55]. The serotype distribution patterns in the subjects with cardiovascular diseases were demonstrated to be totally different compared with healthy subjects. Thus, it might be possible to also use serotype determinations as a means of identifying subjects at risk for developing cardiovascular diseases although additional confirmation of such a relationship is still required.

Although there are a large number of studies attempting to identify the association of the cell surface protein antigens and the pathogenesis of dental caries, the development of molecular methods to analyze for virulence genes is relatively uncommon. In this regard, one of the approaches is RFLP analysis of the *gtf* genes, which is based upon the high diversity of the *gtf* genes [56]. In that method, the 5.2-kb *gtfB* and 4.3-kb *gtfC* genes amplified by PCR are digested with *Bsr*I and *Ssp*I, respectively. Ten and five genotypes were designated based on the digestion patterns for *gtfB* and *gtfC*, respectively. However, there were no correlations found between specific genotypes and the GTF enzymatic activities.

Recently, the *cnm* gene, encoding a 120-kDa cell-surface collagen-binding adhesin of *S. mutans*, was cloned and sequenced [57], which has received attention due to the possible association of dental caries with infective endocarditis [58]. *S. mutans* strains with the *cnm* gene are estimated to be present in approximately 10–20% of individuals. The *cnm*-positive strains possess significantly higher activities for binding type I collagen than the *cnm*-negative strains (Fig. 9.5a). Thus, *S. mutans* strains with *cnm* were predicted to show high virulence for dental caries since type I collagen is also a major organic component of dentin. It was also proposed that the *cnm*-positive strains could bind with higher affinity than the *cnm*-negative strains once the dentin is exposed as caries progresses. In fact, clinical parameters indicate that dental caries in children with *cnm*-positive *S. mutans* in saliva was significantly higher than those with *cnm*-negative *S. mutans* strains as well as *S. mutans*-negative children (Fig. 9.5b).

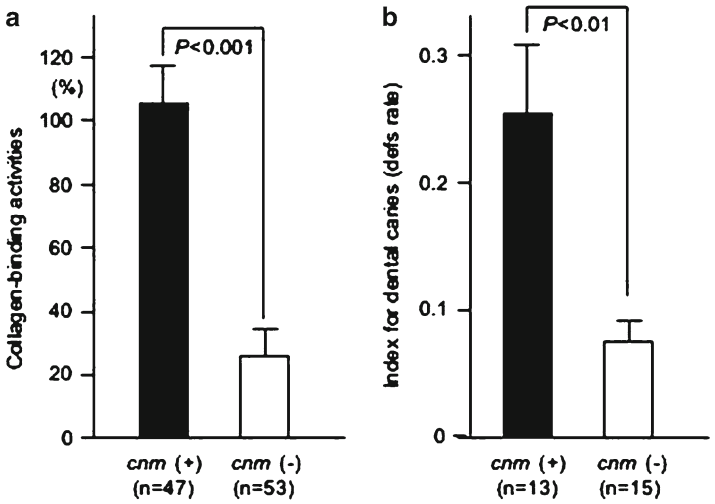

Fig. 9.5 Properties of *S. mutans* strains with *cnm* genes. (**a**) Collagen-binding activity of the strains with or without *cnm* genes when that of strain TW871 as 100%. (**b**) Dental caries index for the subjects harboring strains with or without the *cnm* gene

The *cnm* gene consists of the conserved collagen-binding domain in the 5′-region, followed by a region containing multiple B-repeats, whose length varied among the different strains. The primer set specific for the *S. mutans cnm* gene was constructed based on the nucleotide alignment of the *cnm* gene (cnm-1F 5′-GAC AAA GAA ATG AAA GAT GT-3′ and cnm-1R 5′-GCA AAG ACT CTT GTC CCT GC-3′). The size of the amplified fragments varied from approximately 1,650–1,750 bp due to the number of repeats within the amplicon. The distribution frequency of the *cnm* gene in *S. mutans* strains in the oral cavity is estimated to be approximately 10–20%, with the *cnm*-positive strains showing a predominant distribution among strains with the minor serotypes *f* and *k*.

It was reported that a *S. mutans* strain with defects in the expression of all three GTFs has been isolated [58]. This defect of caused a drastic reduction in its virulence potential for inducing dental caries [40], however, the concomitant decrease in GTF antigenicity was speculated to result in lower susceptibility to phagocytosis by polymorphonuclear leukocytes. This could result in the enhanced survival of such strains in blood compared to GTF-expressing strains. Using the specific nucleotide alignment in *gtf* regions, primer sets specific for detection of similar non-GTF expressing strains were constructed [59]. The detection rate for such strains was shown to be quite low. It has been hypothesized that alterations of cell surface structures of *S. mutans* are considered to be related to the survival in blood as well as the pathogenicity for infective endocarditis [60]. Therefore, further studies focused on the relationship between the cell surface antigens and pathogenesis as well as the development of PCR methods to identify subjects with these highly virulent strains should be considered.

9.6 Transmission Studies

Acquisition of *S. mutans* is considered to be initiated after the first primary tooth erupts, which provides a location for the bacterium to be colonized [61]. The first tooth which erupts is the generally mandibular primary central incisor, which emerges into the oral cavity at an age between 6 and 12 months. The number of erupted teeth increases as children grow, and they face a critical period for colonization at the age between 19 and 31 months called the "window of infectivity" [62], although the speed of colonization could depend on the caries activity of the bacterial population. More recent studies indicate that *S. mutans* can colonize the mouths of predentate infants [63], which indicates the possibility that the predentate children receive *S. mutans* frequently in their mouth and some are transient and some can colonize on the tongue. They are then able to attach to the surface of the first erupting tooth when circumstances are favorable such as with sugar ingestion. The original sources of *S. mutans* have been demonstrated to be mainly their mothers from a large number of studies conducted worldwide. Longitudinal studies regarding the genotypes of *S. mutans* in children demonstrate that most of the initially acquired genotypes generally transmitted from the mothers persist and some are lost and new strains are also acquired. In addition, the sharing of *S. mutans* genotypes between siblings has also been reported, which suggests the possibility of horizontal transfer of strains acquired from mothers between siblings [64]. Furthermore, the transmission of *S. mutans* strains from other family members or other care givers has also been considered.

Saliva is considered to be the major vehicle for oral bacterial transmission, and a high level of salivary *S. mutans* in mothers results in the earlier colonization of the bacterium in their children [65]. In addition, saliva specimens are thought to reflect the composition of the whole oral cavity, whereas dental plaque specimens primarily indicate localized colonization [66]. Studies regarding the transmission of *S. mutans* have been performed by comparison of the isolated strains using various subtyping strategies, such as serotyping, bacteriocin activity profiles, and molecular typings [4]. As for molecular biological methods, CDF techniques commonly employed with *Hae*III digestion, ribotyping, AP-PCR assays, or random-amplified polymorphic DNA (RAPD) analyses are generally used [43, 67]. In addition, the diversity of *S. mutans* strains from children and their mothers was investigated by RFLP of the *gtfB* gene digested by *Hae*III [68].

It should be advantageous to analyze as many strains as possible in a single subject when performing transmission studies. Since there exist time and financial limitations, the number of the estimated genotypes in the populations should be carefully considered when constructing the study design. As for Japanese, approximately 90% of the subjects are estimated to harbor one or two genotypes (average; 1.9 genotypes) [69]. Another study conducted in China demonstrated that 95% of the subjects aged 9–14 years possess one or two genotypes (average; 1.5 genotypes) [70]. Thus, 3–5 randomly selected representative strains should be sufficient for *S. mutans* to be analysis when performing transmission studies.

On the other hand, a study carried out in Sweden showed that only 60% of the subjects between the ages of 20–40 possessed fewer than two genotypes (average; 2.6 genotypes), and the maximum number of the genotypes was shown to be seven [67]. In addition, analysis of the subjects aged 18–29 years held in Brazil showed that a caries-free group possessed one to four genotypes (average; 3.0 genotypes) and that the caries-active group possessed two to eight genotypes (average; 5.5 genotypes). When analyzing these populations, the numbers of the strains are recommended for the study should be as large as possible [71]. In addition, it should be noted that the number of genotypes of *S. mutans* could be influenced by the dental caries status of each individual. AP-PCR analyses revealed that the children with severe dental caries based on the inappropriate usage of nursing bottles showed higher numbers of genotypes as compared with that of caries-free children [72]. This finding suggests that during favorable circumstances, such as sugar exposure, it is easier for new genotypes to be colonized.

The intra-familial transmission rates have been reported by a large number of groups worldwide and could be influenced by many factors such as cultural background, even within similar populations in the same country. Although most of the studies focus on the mother–child transmission of *S. mutans*, there is one study considering father–child transmission [69]. In that study, analysis of 1908 isolates from 76 subjects with 20 Japanese families including children below the age of 12 demonstrated that the transmission ratio from mothers and fathers were demonstrated to be 51.4% and 31.4%, respectively.

9.7 Multilocus Sequencing Typing Approach

Multilocus sequencing typing (MLST) is a generic typing method, employed to date principally, but not solely with bacterial pathogens, which aims to be a robust and portable method for the characterization of bacterial isolates at the molecular level. This method differs from many other approaches for characterization in that it is based explicitly on population genetic concepts [73]. MLST usually employs allele fragments of housekeeping genes approximately 400–600 bp in length and 6–10 loci were selected for sequencing because MLST provides sufficient discrimination for bacterial typing without being subject to diversifying selections which could obscure relationships among isolates. This method was first developed in 1998 in a study of *Neisseria meningitides* [74] and it has been applied for examination of approximately 40 species of microorganisms.

As mentioned above, several genotypic typing methodologies have been used to subtype *S. mutans* including multilocus enzyme electrophoresis (MLEE), ribotyping, and RAPD [64, 67, 71]. More discriminating methods for the subtyping of *S. mutans* include pulsed-field gel electrophoresis (PFGE) [75]. However, these methods differ in their discriminatory abilities power and reproducibility. Therefore, we have developed the MLST method for *S. mutans* typing (Fig. 9.6). Table 9.5 lists the eight housekeeping gene loci applied for a MLST scheme for *S. mutans* [6].

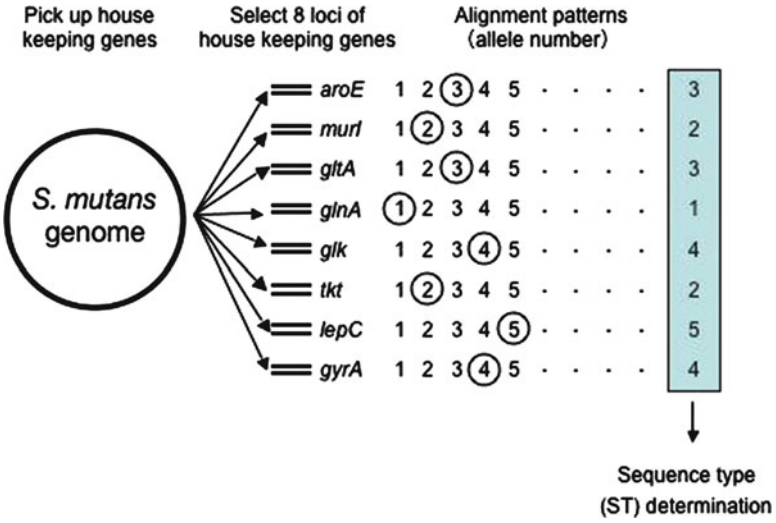
Pick up house keeping genes
Select 8 loci of house keeping genes
Alignment patterns (allele number)
S. mutans genome
aroE 1 2 3 4 5 3
murI 1 2 3 4 5 2
gltA 1 2 3 4 5 3
glnA 1 2 3 4 5 1
glk 1 2 3 4 5 4
tkt 1 2 3 4 5 2
lepC 1 2 3 4 5 5
gyrA 1 2 3 4 5 4
Sequence type (ST) determination

Fig. 9.6 Strategy for multilocus sequence typing (MLST) method of *S. mutans*

Table 9.5 Characteristics of housekeeping genes in *S. mutans* MLST scheme

Genes	Gene locus tags	Gene products	Number of allele identified[a]
tkt	Smu.291	Transketorase	14
glnA	Smu.364	Glutamine synthase	18
gltA	Smu.365	Glutamate synthase	26
glk	Smu.542	Glucose kinase	19
aroE	Smu.778	Shikimate 5-dehydrogenase	22
gyrA	Smu.1114	DNA gyrase subunit A	17
murI	Smu.1718	Glutamate racemase	19
lepC	Smu.1874	Signal peptidase I	25

[a]Number was determined based on the analyses of total of 238 strains from 142 subjects

Initially, the internal fragments of the housekeeping genes were amplified and their nucleotide sequences determined (GenBank accession numbers AB281702-AB282509 and AB427220-AB428307). The sequences for each allele are compared with those in nonredundant databases (http://pubmlst.org/oralstrep/) and allele numbers were assigned for each strain, which defines the allelic profile. Finally, the sequence types (STs) for each strain are assigned. Numbers of the alleles for eight kinds of housekeeping genes are between 14 and 26 and STs 1–108 are assigned for 238 strains from 142 subjects at present [4, 6].

Figure 9.7 shows a phylogenetic tree based on hierarchical clustering of MLST results with 108 STs from 238 strains isolated in Japan and Finland. This result indicated that *S. mutans* contains a diverse population. This method was proven to theoretically distinguish more than 1.2×10^{10} sequence types. The serotype *c* strains

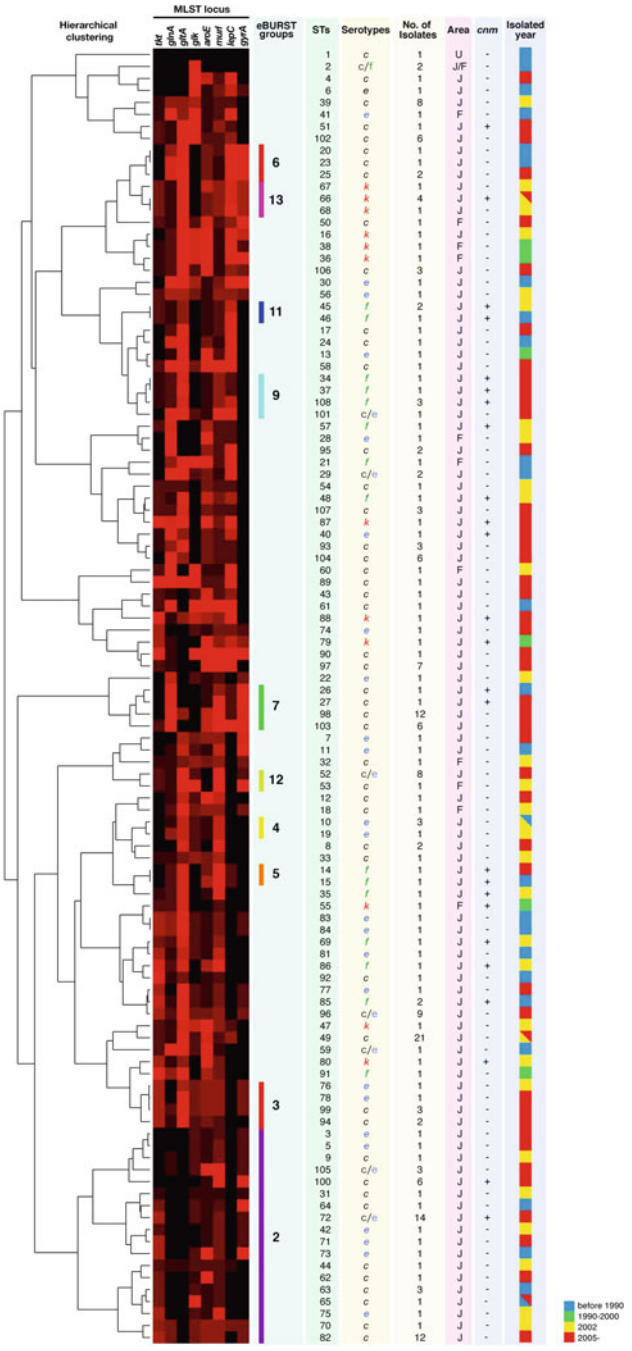

Fig. 9.7 Cluster analysis of ST profiles and the relationship among serotypes, year of isolation, and areas of isolation. Phylogenetic tree constructed based on the 164 strains isolated from Japan and Finland using CLUSTER3 software (http://bonsai.ims.u-tokyo.ac.jp/%7Emdehoon/software/cluster/software.htm) and Java TreeView (http://jtreeview.sourceforge.net/). Area—U, USA; J, Japan; F, Finland

are shown to be widely distributed in the tree, whereas the serotype *e*, *f*, and *k* strains were differentiated into clonal complexes, suggesting that the original ancestral strain of *S. mutans* was serotype *c*. Although no geographic specificity was identified, the distribution of the *cnm* gene was demonstrated to be clearly evident.

The superior discriminatory capacity of this MLST method for *S. mutans* may have important practical implications. Although various kinds of subtyping methods have been applied for transmission studies of *S. mutans*, the high discriminatory power gained by the MLST method is considered to result in greater sensitivity. Using MLST, 20 Japanese mother–child pairs whose children were between 2 and 10 years of ages showed that transmission could be observed with 70% of the pairs [4]. The MLST method could be applied for various epidemiological studies, which possibly could lead to grouping of the virulent strains of *S. mutans* into specific clusters to aid clinical assessment in the near future.

9.8 Summary

In summary, recent development of molecular biological techniques enables the detection of target bacterial species and their virulence genes without direct isolation of the strains. As for *S. mutans*, there have been a large number of such approaches developed. Identification of *S. mutans* followed by speciation of the highly virulent strains for dental caries as well as other diseases could be one of the powerful tools for clinical interventions in the future. Accumulation of data from clinical studies using molecular biological techniques might lead to the development of novel relevant systems for clinical use.

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Chapter 10
Genotyping of Periodontal Anaerobic Bacteria in Relationship to Pathogenesis

Masae Kuboniwa and Atsuo Amano

10.1 Introduction

Adult periodontitis is a chronic inflammatory gum disease that results from the complex actions of a small subset of periodontal pathogens. Although the central cause of periodontitis is loss of healthy balance between microbial virulent agents and host immunity in host-parasite interactions, there are marked differences in the progression rate and severity of this infectious disorder, as well as response to therapy among patients. Thus, periodontitis is not considered to be a homogeneous disease, but rather intricately influenced by host susceptibility differences as well as diversities in virulence among the harbored organisms. Indeed, *Porphyromonas gingivalis,* a bona fide periodontal pathogen associated with various forms of marginal periodontitis, can be present in periodontal pockets undergoing destruction as well as in healthy gingival margins. Clonal heterogeneity of subpopulations with both high and low levels of pathogenicity has been suggested to exist among periodontal pathogens harbored by individuals with negligible, slight, or even severe periodontal destruction. Therefore, specific virulent clones of the pathogens may be the cause of advanced and/or aggressive periodontitis.

Such aspects are not pathognomonic for only periodontitis, as they are also commonly observed features of various infectious diseases caused by a wide variety of pathogens in humans. Recent technical innovations have provided various genomic

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tools for identification of microorganisms, as well as evaluation of the presence of virulence factors and antibiotic resistance determinants. Genotyping assays of periodontal pathogens are expected to become useful methods for periodontal examinations and diagnosis. However, those will require additional refinements to elucidate how the degree of clonality of particular periodontal pathogens influences outcome and results interpretation. This chapter describes currently available assay methods, including those for the most advanced microbiome analyses, which are used to investigate disease-causing bacterial flora and genotypic variations of periodontal pathogens, as well as findings from past studies that enable the estimation of specific virulent phenotypes and genotypes related to periodontitis.

10.2 Classification of Bacteria in Pre-Genomic and Post-Genomic Eras

Screening for the existence of particular species recognized as periodontal pathogens is the first step of diagnosis and usually performed prior to evaluation of virulent subspecies. Despite its eminent practical significance for identification, diagnosis, and diversity surveys, bacterial species definition remains a very difficult issue for researchers. In 1987, it was proposed that bacterial strains showing >70% DNA-DNA hybridization and sharing characteristic phenotypic traits should be considered as the same species, which is still the most recent definition of bacterial species officially utilized. On the other hand, advances in sequencing techniques have introduced other markers for bacterial classification, such as 16S ribosomal RNA (rRNA), a molecule that is ubiquitous in bacterial genomes. Sequence similarities of 16S rRNA genes have been found to be highly correlated with DNA hybridization, with a roughly 97% 16S rRNA sequence identity considered to correspond to the 70% cutoff level in DNA-DNA reassociation, and widely used for estimation of evolutionary history and taxonomic assignment of individual organisms. For this purpose, sequence identity ranging from 90 to 95% is sometimes used to define genera, and a range of 97–98.7% generally used to define a species or phylotype. However, this phylogenetic definition has some disadvantages. First, the 16S rRNA typing method lacks resolution below the species level, though strains may be distinguished at the level of 99% pair wise sequence identity. Second, a phylogenetic definition has some exceptions that include the *Bacillus* genus, for which the 16S rRNA sequences from phenotypically distinct species differ at only a few bases. In contrast, considerable intragenomic variations of 16S rRNA genes were found in 24 species according to the current GenBank database and, when compared with the 16S rRNA-based threshold for operational definition of species, the diversity was borderline (between 1 and 1.3%) in 10 species and >1.3% in 14 species [1]. Thus, taxonomic classification using the 16S rRNA-based operational threshold might misclassify a number of species, leading to under- or overestimation of the diversity of a complex microbiome.

10.3 Metagenomic Analysis of Oral Microbiome

10.3.1 Microbiome Study Using 16S Ribosomal RNA Gene Clone Library

Despite the taxonomic dilemma described in the former section, a ribosomal RNA approach remains the principal tool to study microbial diversity. During the last decade, microbiome studies of clinical specimens, including saliva, supragingival plaque, subgingival plaque, and tongue coating, were performed by constructing 16S rRNA gene clone libraries. However, they may have considerable biases related to the polymerase chain reaction (PCR) primers used and the relative inefficiency of DNA extraction techniques. So-called "universal" PCR primers can introduce bias into analysis of a species composition of clone libraries, because of mismatches between the primer and target organism sequences. Thus, a combined use of multiple universal primer sets, multiple DNA extraction techniques, and deep community sequencing is recommended for minimizing such biases and recovering substantially more species than reported in prior studies.

10.3.2 Microbiome Study Using 16S Ribosomal RNA Gene Hypervariable Tag Sequence

Presently, metagenomic studies rely on the utilization and analysis of reads obtained using next-generation sequencing (NGS), such as 454 pyrosequencing, and Illumina and SOLiD sequencing, to replace conventional Sanger sequencing. NGS has markedly accelerated multiple areas of genomics research, enabling experiments that previously were not technically feasible or affordable. However, the sequences are much shorter, thus new methods are necessary to identify microbes from short DNA tags.

The 16S rRNA gene in bacteria consists of conserved sequences interspersed with variable sequences that include nine hypervariable regions (V1–V9), whose lengths range from approximately 5 to 100 bases. Individual reference databases for the specific hypervariable regions 3, 6, and 9 of 16S rRNA genes (RefHVR_v3, RefHVR _v6, and RefHVR _v9, respectively) were created by excising *in silico* the appropriate sections of full-length sequences from the SILVA database (http://www.arb-silva.de/), and are available at the Visualization and Analysis of Microbial Population Structures (VAMPS) Web site (http://vamps.mbl.edu/resources/databases.php). The taxonomies assigned to hypervariable regions three and six were compared with those assigned to the full-length 16S rRNA sequences, and the hypervariable region tags and full-length ribosomal RNA sequences were found to provide equivalent taxonomy and measures of relative abundance of microbial communities. In addition, the numbers of different phylotypes and their relative

abundance in saliva and supragingival plaque samples from healthy adult populations were determined using V6 hypervariable region pyrosequencing analysis, with an estimated 19,000 phylotypes identified, a number considerably higher than previously estimated [2]. Very recently, the ~82 base segment in V5 has been shown to be a short region which provided reliable identification of oral bacteria by Illumina technology. However, phylum *Bacteroidetes* was detected at a lower rate by this method [3].

10.3.3 Terminal Restriction Fragment Length Polymorphism

Terminal restriction fragment length polymorphism analysis, a quantitative molecular PCR technique, was developed for rapid analysis of microbial community diversity in various environments. With this method, one of the two primers is fluorescently labeled at the 5′ end and used to amplify a selected region of bacterial genes encoding 16S ribosomal RNA from the total community of DNA. The PCR product is digested with restriction enzymes and the fluorescently labeled terminal restriction fragment precisely measured using an automated DNA sequencer. This analysis has been applied to assess oral microbial communities in subgingival specimens for monitoring changes after clinical treatment [4], as well as those in saliva obtained from oral malodor patients [5].

10.3.4 Microbiome Study Using Whole-Genome Shotgun Sequencing

As described in the former section, 16S rRNA gene-based sequencing can detect predominant members of a microbial community, though it may not detect rare members of a community with divergent target sequences. Primer bias and the low depth of sampling cause some of these limitations. To overcome the limitations of single gene-based amplicon sequencing, whole-genome shotgun sequencing has emerged as an attractive strategy for assessing complex microbial diversity in mixed populations.

One key issue of whole-genome sequencing strategies is the requirement for sufficient amounts of input genomic DNA for comprehensive studies based on metagenomics. Whole-genome amplification represents an effective technology for enabling whole-genome shotgun sequencing of limited amounts of total DNA in the precious samples. However, the potential of whole-genome amplification to co-amplify contaminating host (human) DNA poses a significant problem, while such host DNA co-amplification may also overwhelm the bacterial DNA sequence data in the sample. Different subtraction strategies for human DNA sequences are needed to minimize this possible blockade.

10.3.5 Third-Generation Sequencing—Single-Molecule Sequencing Technologies

Third-generation sequencing technologies, including real-time single-molecule DNA sequencing and nanopore-based sequencing, may drastically decrease sequencing time, reduce costs, and streamline sample preparation. Real-time single-molecule sequencing developed by VisiGen uses DNA polymerase modified with a fluorescent donor molecule. Pacific Biosciences performs another type of single-molecule sequencing, which uses phospholinked fluorescently labeled dNTPs. Nanopore-based sequencing monitors the passage of DNA molecules through nanopores 2–5 nm or greater in diameter, by which kilobase length polymers (single-strand genomic DNA or RNA) can be identified and characterized without amplification bias. Third generation instruments can sequence a mammalian genome for ~$1,000 in ~24 h, however, the large amounts of sequence data outputted will cause a bioinformatics challenge for the clinical laboratory performing the test. In addition to data processing, the interpretation of sequencing results will require further characterization of the genomic variation, as described in the Sect. 10.4.6

10.3.6 Pan-Genome Analysis

The development of efficient and inexpensive genome sequencing methods has revolutionized the study of human bacterial pathogens. However, the sequence of a single genome does not reflect how genetic variability drives pathogenesis within a bacterial species. Tettelin et al. analyzed eight genomes considered to be representative of the serotype diversity among *Streptococcus agalactiae* strains to answer the question of how many genomes are needed to fully describe a bacterial species [6]. Analysis of these genomes and those in available databases showed that *S. agalactiae* species can be described by a pan-genome consisting of a "core genome" shared by all isolates, accounting for approximately 80% of any single genome, plus a "dispensable genome" consisting of partially shared and strain-specific genes.

Recently, the pan-genome concept has been extended to higher taxonomic units. The size of the bacterial pan-genome was estimated based on the frequency of occurrences of genes among 573 sequenced genomes, with three distinct pools of gene families; core, character and accessory gene families, characterized (Fig. 10.1). The results indicate that the pan-genome of the bacterial domain is of infinite size and that approximately 250 genes per genome belong to the extended bacterial core genome [7].

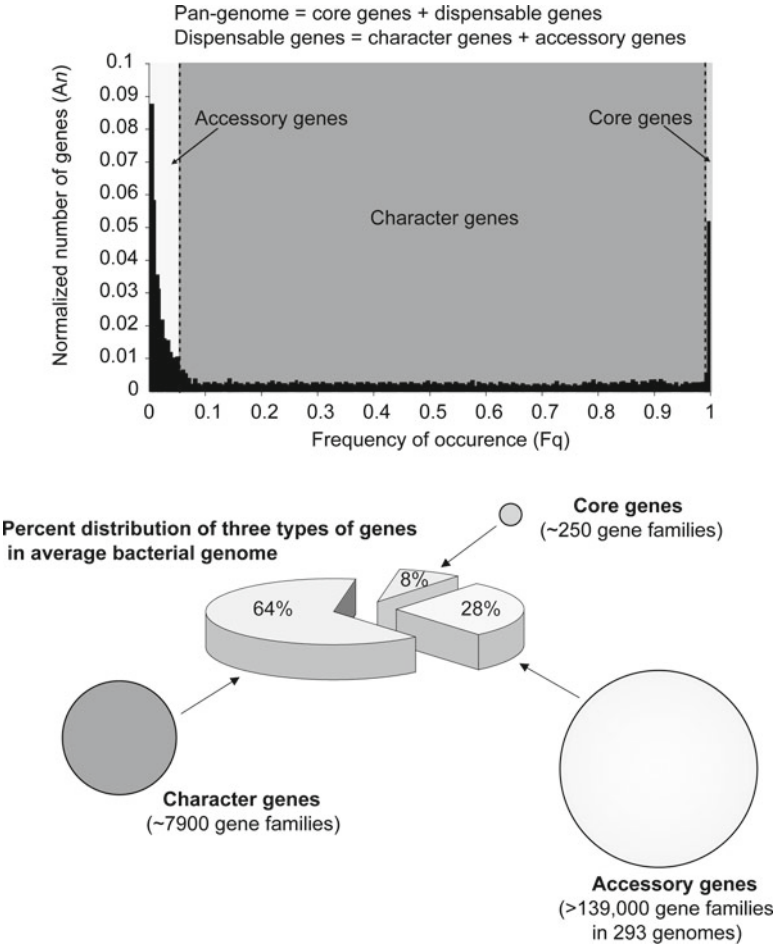

Fig. 10.1 The bacterial pan-genome. (**a**) A total of 15,000 genes were sampled to determine their frequencies of occurrence among 293 genomes (Lapierre Trends in Genetics). A Bitscore >50 obtained using a basic local alignment search tool (BLAST) was used to classify a gene as present in the target genome and a member of the same gene family. Each bar corresponds to the normalized number of genes [*n* genes at Fq(*x*)/15,000] having the indicated frequency (Fq) of occurrence (present in *n* other genomes/total number of genomes − 1). Genes without any homologs (Fq = 0) represent accessory genes, whereas genes present in 292 other genomes (Fq = 1) represent strict core genes. Parts of the histogram that mainly contribute to the extended core genes, as well as character and accessory genes are indicated. (**b**) Each gene found in the bacterial genome represents one of three pools. Genes found in all but a few bacterial genomes comprise the extended core of essential genes that encode proteins involved in translation, replication, and energy homeostasis; character genes represent genes essential for colonization and survival in particular environmental niches; and the accessory gene family, comprising a pool of apparently infinite size, contains genes that can be used to distinguish strains and serotypes, though the function of most genes in this category is unknown

10.3.7 "Human Microbiome Project" and "Genomic Encyclopedia of Bacteria and Archaea"

The human microbiome refers to the community of microorganisms that populate the human body. The National Institutes of Health launched an initiative that focuses on describing the diversity of microbial species associated with health and disease, and the results from an initial reference genome sequencing of 178 microbial genomes, including those of oral microorganisms, are presented at The Human Microbiome Project Web site (http://www.hmpdacc.org/) [8]. On the other hand, The Genomes Online Database (GOLD; http://www.genomesonline.org/) released 1,291 completed bacterial and archaeal genome sequences, most of which were chosen for sequencing on the basis of their physiology. As a consequence, the perspective provided by the currently available genomes is limited by a highly biased phylogenetic distribution. Very recently, genomes of 56 culturable species of bacteria and archaea have been selected and sequenced to maximize the phylogenetic coverage. Analysis of these genomes demonstrated pronounced benefits in diverse areas, including the reconstruction of phylogenetic history, the discovery of new protein families and biological properties, and the prediction of functions for known genes from other organisms. To utilize a phylogeny-driven "Genomic Encyclopedia of Bacteria and Archaea" would be advantageous to derive maximum knowledge from genome sequences yet to be revealed [9].

10.4 Genotyping Methods to Distinguish Clonality of Periodontal Pathogens

Studies of pathogens require uniform and reproducible nomenclature schemes, and molecular typing systems are able to distinguish among epidemiologically unrelated isolates by characterizing the genetic variations in the chromosomal DNA of bacterial species. Several of the DNA-based typing methods described below have been used for characterizing periodontal pathogens during the previous 10 years. Among these methods, multi-locus sequence typing (MLST) and PCR analysis for detection of target genotypes have been the most commonly utilized to discriminate isolates below the species level (Fig. 10.2).

10.4.1 Mobility Shift Assays

Electrophoretic mobility shift assays (EMSA), including denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), temperature sweep gel electrophoresis (TSGE), and heteroduplex analysis (HA), were

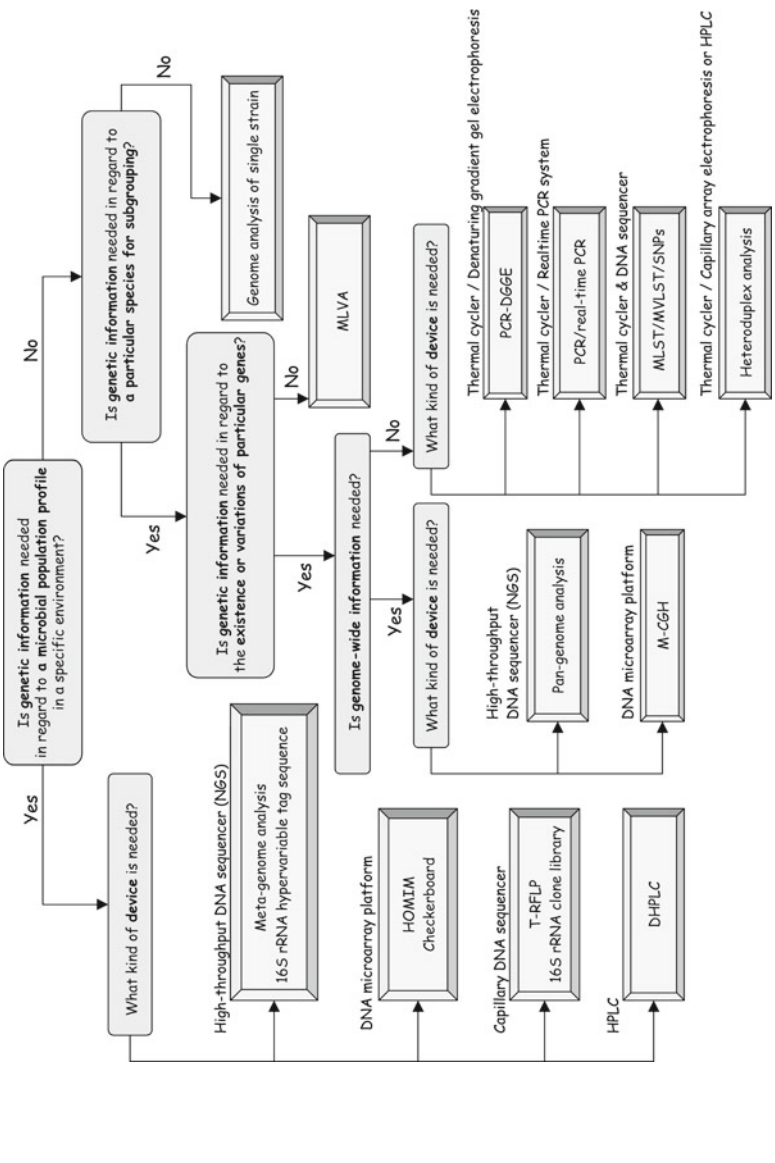

Fig. 10.2 Flowchart for selection of molecular typing method. Based on the purpose and available device, appropriate ways to perform molecular typing are presented

originally developed as tools for detection of genetic mutations in cancer genomes. However, they have also been found suitable for screening of virulent phylotypes in oral biofilm as well. In EMSA, DNA fragments of the same length, but with different base-pair sequences, can be separated. Even a single-base change in a sequence can be resolved, which provides PCR-EMSA with great potential to identify closely related species based on 16S rRNA sequence divergence. In addition to being applied to the broad-range analysis of complex microbial communities [10, 11], the method can also be used to detect specific bacterial groups by altering the primer targets or by using a nested PCR approach. Moreover, denaturing high performance liquid chromatography (DHPLC), an electrophoresis-independent mobility shift assay, has been applied to the monitoring of microflora [12].

10.4.2 Multi-Locus Sequence Typing

The ability to accurately characterize species is reliant on strain typing methods used to distinguish among isolates of the same species, and is usually accomplished using one or more DNA-based methods. Multiple locus sequence typing (MLST), a phylogenetic approach designed to infer the relatedness among strains of various bacterial pathogens, has been used in several etiologic studies of periodontal diseases. With this method, 7–10 housekeeping genes are generally chosen, depending on the species of interest, which are then sequenced and the resultant individual gene sequences linked in tandem, or concatenated, prior to phylogenetic analysis. The resulting data is storable in a digital format so that worldwide coverage of pathogen diversity can eventually be achieved (http://pubmlst.org/). MLST has been performed in studies of *P. gingivalis, A. actinomycetemcomitans*, and *T. denticola*. However, this method seems to be incapable of linking strains to pathogenesis, thus direct genotyping of virulence related genes is still necessary for clustering of virulent strains [13].

MLST involves sequencing portions of 7–10 housekeeping genes, thus the samples comprise only about 0.1–0.2% of a microbial genome (Fig. 10.3, Table 10.1). As a result, it is not surprising that a large number of isolates appear to be identical when using such small fractions of their genomes. The question remains whether all or many of those isolates are actually different from each other. It seems likely that higher resolution methods could usefully distinguish them and whole-genome information may provide such a desired increase in resolution, as described in the "Pan-genome analysis as a strain typing solution" section.

10.4.3 Multi-Virulence-Locus Sequence Typing

The multi-virulence-locus sequence typing (MVLST) scheme was originally developed for subtyping *Listeria monocytogenes* [14]. Internal fragments (ca. 418–469 bp) of three virulence genes and three virulence-associated genes were sequenced, and then multiple DNA sequence alignment identified a total of 28

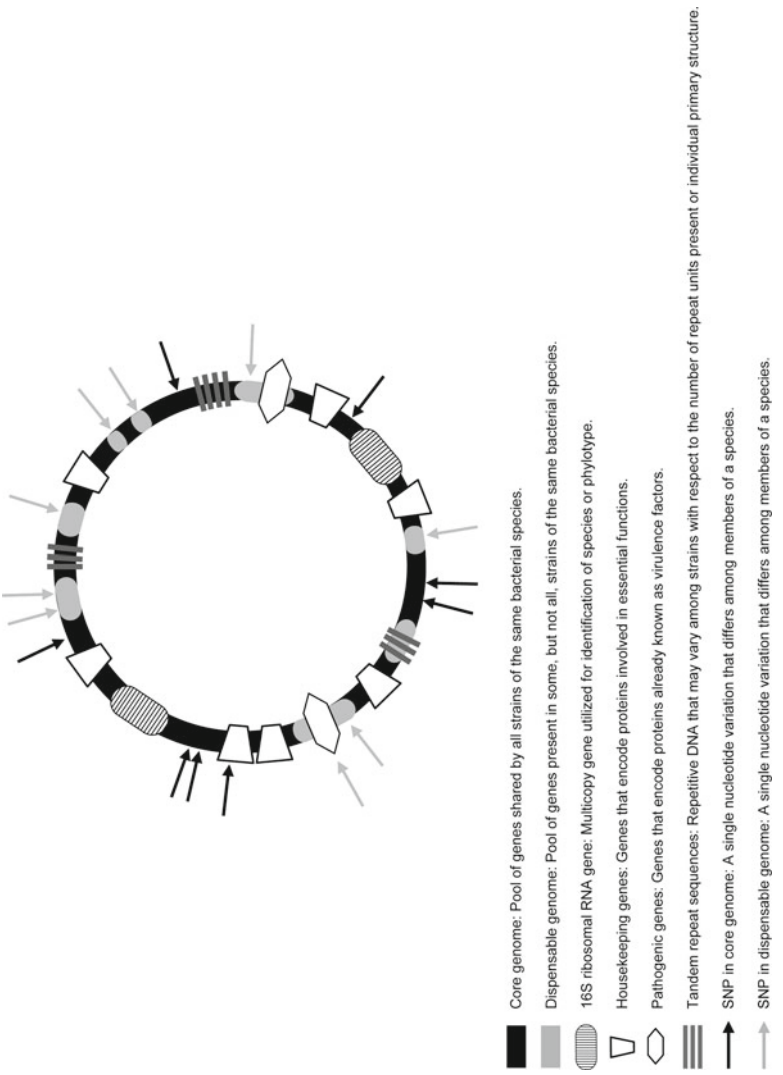
Core genome: Pool of genes shared by all strains of the same bacterial species.
Dispensable genome: Pool of genes present in some, but not all, strains of the same bacterial species.
16S ribosomal RNA gene: Multicopy gene utilized for identification of species or phylotype.
Housekeeping genes: Genes that encode proteins involved in essential functions.
Pathogenic genes: Genes that encode proteins already known as virulence factors.
Tandem repeat sequences: Repetitive DNA that may vary among strains with respect to the number of repeat units present or individual primary structure.
SNP in core genome: A single nucleotide variation that differs among members of a species.
SNP in dispensable genome: A single nucleotide variation that differs among members of a species.

Fig. 10.3 Genomic coverage of genetic typing methods. The core genome includes genes that encode proteins involved in essential functions, such as replication, transcription, and translation. The dispensable genome includes genes that encode proteins that facilitate organismal adaptation. Coverage by 16S ribosomal RNA (rRNA), housekeeping genes for multilocus sequence typing (MLST), pathogenic genes for multi-virulence-locus sequence typing (MVLST), tandem repeat sequences for multilocus variable number of tandem repeats analysis (MLVA), and single-nucleotide polymorphisms (SNPs) is also depicted

Table 10.1 Genomic coverage of genetic typing methods

Typing method	Genetic loci	Genomic coverage rate (%)	Classification level
16S rRNA	1 locus (various copy number)	0.062[a]	Domain, phylum, class, order, family, genus, (species)
MLST	~10 loci	0.26[b]	Clonal complex (subspecies)
SNPs	~100 loci	~2[c]	Haplotype
Pan-genome	Whole genome	100	All non-clonal genetic variations

[a]Calculated based on genome size (2,354,886 bp) and 16S rRNA size (1,474 bp) in *Porphyromonas gingivalis* ATCC33277

[b]Calculated based on genome size (2,354,886 bp) and sum of MLST target region size [*ef-tu* (650 bp), *ftsQ* (566 bp), *hagB* (560 bp), *gpdxJ* (501 bp), *pepO* (642 bp), *mcmA* (562 bp), *dnaK* (659 bp), *recA* (642 bp), *pga* (693 bp), *nah* (683 bp)] in *P. gingivalis* ATCC33277 [28]

[c]Calculated based on genome size (~4.8 Mb) and SNP gene fragment size (~89 Kb) in *Salmonella enterica* serovar Typhi [29]

unique sequence types. Comparison of MVLST results with those of automated EcoRI- ribotyping (RT) and pulsed-field gel electrophoresis (PFGE) with *Apa*I enzymatic digestion showed that MVLST was able to differentiate strains that were indistinguishable by RT or PFGE. Furthermore, a comparison of MVLST with housekeeping gene-based MLST analysis showed that MVLST provided higher discriminatory power for some strains than MLST, while cluster analysis based on the intragenic sequences of the selected virulence genes indicated a strain phylogeny closely related to serotypes and genetic lineages. Thus, MVLST may improve the discriminatory power of MLST and become a convenient tool for studying the molecular epidemiology of periodontal pathogens.

10.4.4 Single Nucleotide Polymorphisms

Single nucleotide polymorphisms (SNPs) were originally developed for use in humans and then applied to bacteria. SNPs have recently been utilized to differentiate *Bacillus anthracis* clinical samples that were collected from a disease outbreak and to propose a *Mycobacterium tuberculosis* typing scheme. Furthermore, a set of 16 SNPs was reported able to differentiate all epidemic clones and outbreak strains of *L. monocytogenes* [15]. SNPs can be a powerful tool owing to their provision of greater genomic coverage as compared with other classification methods (Fig. 10.3, Table 10.1). However, their use is limited and their potential for more general use in bacterial population genetics remains unproven.

10.4.5 Multiple-Loci Variable Number of Tandem Repeats Analysis

A large number of bacterial genes or intergenic regions contain loci of repetitive DNA, which may vary among strains with respect to their individual primary structure or the number of repeat units present [16]. This has implications for both the

techniques used to determine the number of repeats and the level of variability. In addition, tandem repeats can be part of coding regions or intergenic, and may play a direct role in adaptation to the environment, thus the observed evolution rates may differ. For these reasons, the choice of a variable number of tandem repeats is important for this type of analysis. Although reasonable discrimination can be achieved with the typing of 6–8 markers, particularly with species with high genomic diversity, it may be necessary to type 20–40 markers in order to cluster pathogenic strains in monomorphic species. A variable number of tandem repeat data sets for the *P. gingivalis* strains ATCC33277 and W83 are currently available online (http://minisatellites.u-psud.fr/ASPSamp/base_ms/bact.php).

10.4.6 Pan-Genome Analysis as Strain Typing Solution

Comparative genomics analyses between multiple genomes of individual species have revealed extensive genomic intraspecies diversity [17]. Some intraspecies molecular evolutionary mechanisms, including point mutation, gene duplication, gene loss, and recombination, have been reported to contribute to bacterial genome diversity. Interspecies mechanisms, such as phage infection and plasmid acquisition, as well as population dynamic mechanisms including bottleneck and selective sweep are also known as forces that shape bacterial genomes. For these reasons, one genome sequence is inadequate to describe the complexity of species, genera, and their interrelationships. The term "pan-genome" denotes the set of all genes present in the genomes of a group of organisms, usually a species. As described in the former section, a pan-genome includes genes that exist in only a single organism (accessory genes), in the genomes of a few members of the group (character genes), or in all genomes of the group (core genes). A group of accessory genes and character genes is described as distributed genes or dispensable genes. Very recently, Hall et al. [18] have developed a novel strain typing method termed neighbor grouping (NG), which is based on core gene distances and the presence or absence of distributed genes. NG defines a pair of genomes as valid neighbors if they are significantly more closely related than an average pair of genomes. According to their report, the results of NG analyses are entirely consistent with those of MLST, while NG provides additional discriminatory power.

10.4.7 Microarray-Based Comparative Genomic Hybridization

Microarray-based comparative genomic hybridization (M-CGH) techniques have been used to characterize the extensive intraspecies genetic diversity found among bacteria at the whole genome level. Very recently, M-CGH has been performed to estimate the whole genomic diversity of representative *P. gingivalis* strains [19].

In that study, the relatedness of the strains to one another shown by this analysis was reported to be highly similar to their relatedness based on ribosomal operon intergenic spacer region sequence analysis, while a correlation was also observed between the genome contents and disease-associated phenotypes of the strains.

10.4.8 In Situ Oligonucleotide Probes

Prokaryotic cells can be identified without cultivation by applying fluorescence in situ hybridization (FISH) with ribosomal RNA targeted oligonucleotide probes. Generally, these probes are 15–25 nucleotides in length and labeled covalently at the 5′ end with a fluorescent dye. Specifically stained cells are detected via epifluorescence microscopy or flow cytometry. This technique has become the method of choice for reliable and rapid identification of microorganisms in environmental and medical samples, such as dental plaque. The large online database "Probebase" provides an encompassing overview of published probes (http://www.microbial-ecology.net/probebase/).

Flow cytometry is a routine method used for cellular biology studies, though its application to prokaryotic cells up to this point has been rather limited, mainly because of the difficulty in interpreting signals from very small objects. However, studies that use flow cytometry techniques for application to environmental microbiology have been increasing in recent years. Although bacteria are relatively difficult to analyze and differentiate by flow cytometry, owing to their small cell size (so-called diminutiveness) and mostly similar morphologies, flow cytometry techniques are especially promising due to their high-throughput capacity and ability for interrogation at the single-cell level. Recent technical advances have simplified flow cytometry instrument handling, improved cell sorting capabilities, and introduced multi-parametric measurements, and it is now possible to interrogate microbial communities using two or three different fluorescent dyes that target specific biomolecules and physiological processes.

10.4.9 Pattern-Based Technologies

Pattern-based technologies, such as restriction fragment length polymorphism (RFLP), multi-locus enzyme electrophoresis (MLEE), arbitrarily primed PCR (AP-PCR) for randomly amplified polymorphic DNA analysis (RAPD), pulse-field gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP), have been used for characterizing periodontal pathogens including *P. gingivalis*, *A. actinomycetemcomitans*, and *T. denticola*. However, it should be noted that patterns produced in different laboratories can only be compared if very strict quality standards are followed.

10.4.10 PCR Analysis for Detection of Target Genes

The identification of bacterial species or detection of particular virulence related genes using molecular techniques, such as conventional polymerase chain reaction (PCR), provide binary presence or absence data, while other methods, such as checkerboard DNA–DNA hybridization or real-time polymerase chain reaction (RT-PCR), provide quantitative data [20, 21]. In addition, PCR analysis has also been used for genotyping of virulence-related genes in *P. gingivalis* and *A. actinomycetemcomitans*, as described in the next section.

10.5 Clonal Variations of Bacterial Molecules Related to Bacterial Virulence Diversity

10.5.1 Variations of P. gingivalis Fimbriae

Fimbriae are thin, filamentous, proteinaceous surface appendages (hair-like organelles) that protrude from the surface of a number of different bacterial species, and are especially prominent on Gram-negative bacteria where they are anchored within the outer membrane [22]. *P. gingivalis* expresses two distinct fimbriae types on its cell surface; one of which is composed of a subunit protein (named FimA or fimbrillin) encoded by the *fimA* gene and termed long, or major fimbriae, while the other consists of a subunit Mfa protein encoded by the *mfa1* gene and termed short, minor, or Mfa fimbriae (henceforth referred to as simply long and short fimbriae, respectively). Long fimbriae are a critical factor for colonization of *P. gingivalis* in subgingival regions, as they promote both bacterial adhesion to and invasion of targeted sites. The *fimA* gene is monocistronic and exists as a single copy in the chromosome of *P. gingivalis*. *P. gingivalis fimA* genes have been classified into six variants (types I to V and Ib) on the basis of their different nucleotide sequences [22].

We developed a conventional PCR assay method using *fimA* type-specific primer sets to differentiate the *fimA* genotypes of the organisms present in saliva and dental plaque samples [22]. With this method, various researchers have investigated the prevalence and distribution of *fimA* genotypes in subjects with different periodontal conditions in various geographical locations, including Japan, China, Germany, Norway, the Netherlands, Switzerland, Brazil, and Mexico. From those epidemiological and clinical studies, it can be derived that the type II *fimA* genotype is most prevalent in periodontitis patients, while the second most prevalent has been variably found to be type IV or Ib, depending on the ethnic population studied. Conversely, types I and III *fimA* are more prevalent in non-periodontitis subjects. As for clinical significance, type II clones are most frequently associated with advanced chronic periodontitis with deep probing pocket depth, and severe forms such as aggressive periodontitis and refractory periodontitis. In addition, type II clones seem to be related to marginal periodontitis in Down's syndrome and mentally disabled populations.

P. gingivalis has been reported to contribute to such systemic conditions as cardiovascular diseases and diabetes mellitus by direct oral-hematogenous spreading of

bacteria. Types II and IV clones were detected in atheromatous plaque specimens collected from patients undergoing cardiovascular surgery more frequently than the other types [23], while type II clones were also shown to be a participation factor in marginal periodontitis associated with type II diabetes mellitus. On the other hand, diabetes glycemic level may be affected by the persistence of *P. gingivalis*, especially type II clones, in periodontal pockets after professional periodontal treatment [24]. Furthermore, type II fimbriae were shown to most efficiently mediate bacterial adhesion to and invasion of host cells, as compared to those of the other types. Following invasion of cells, intracellular *P. gingivalis* organisms with type II fimbriae were found to clearly degrade integrin-related signaling molecules such as paxillin and focal adhesion kinase, thus disabling cellular migration and proliferation. These events are considered to be an integral part of the bacterial strategy for persistence in periodontal tissues. Together, these findings strongly suggest that clonal variations of long fimbriae are related to bacterial infectious traits that influence disease development and deterioration.

10.5.2 Variations of A. actinomycetemcomitans Leukotoxin

Leukotoxin (Ltx) is one of the virulence factors of *A. actinomycetemcomitans* and likely linked to aggressive periodontitis. Ltx is assumed to contribute to the severity of periodontal disease by disrupting local defense mechanisms, and has also been correlated to disease onset and progression. All isolates of *A. actinomycetemcomitans* exhibit the *ltx* operon, which has been classified into three genotypes based on differences in Ltx expression in the representative strains ATCC33384, Y4, and JP2 [25]. Those genotypes were also shown to be related with the expression efficiency of Ltx, i.e., lower (ATCC33384), moderate (Y4), and high (JP2) levels of expression with apparent toxic diversity shown in chromium release assays using monocytes. The Ltx operon of isolates with high titers of leukotoxin production (JP2-like) presents a deletion of 530 bp, resulting in 10–20 times greater levels of Ltx expression than low leukotoxic strains. In addition, the truncated structure of the promoter was shown to drive Ltx expression, while the levels of toxin expressed by 15 strains of *A. actinomycetemcomitans* were found to be correlated with the truncated structure of the *ltx* promoter.

10.5.3 Variations of A. actinomycetemcomitans Cytolethal Distending Toxin

Cytolethal distending toxin (Cdt) is a newly described virulence factor produced by *A. actinomycetemcomitans* that disturbs the cell cycle (G2 arrest) and is involved in cellular proliferation. The *cdt* gene is encoded by three genes, designated as *cdtA*, *cdtB*, and *cdtC*, which are organized in an apparent operon. The *cdt* operon has been classified into four genotypes using a PCR method, which are correlated with various serotypes, with *cdt* genotype 1 found only in serotype b strains, *cdt* genotype 2 only in serotype a and b strains, *cdt* genotype 3 only in serotype c and f strains, and *cdt*

genotype 4 only in serotype c strains [26]. In addition, the *cdt* operon was classified into six restriction fragment length polymorphism (RFLP) types based on *Hind*III-digested genomic DNA [27]. However, there was no strict correlation shown between RFLP type and the cyto-distending activity of each strain. Groups with RFLP types I, II, and IV showed medium cyto-distending activities, while the activities of those with types III, V, and VI varied widely among each type, though types V and VI seemed to have greater cyto-distending activities than the others. These findings suggest that *cdt* genotype may have a relationship with aggressive periodontitis, though the correlation between *cdt* alleles and aggressive periodontitis remains to be defined.

10.6 Conclusion

Development of a useful genotyping testing tool for periodontal pathogens is necessary for therapeutic use. Future dentistry-related research will certainly produce such bacterial testing tools for periodontal diagnosis, as well as medication and treatment for affected individuals. However, additional efforts are required to investigate the exact relationship between genotypic variation and bacterial pathogenicity in periodontitis. Genomic variations of the long fimbria structures of *P. gingivalis* seem to be related to periodontitis initiation and progression. Recently, in addition to the whole genome sequence of *P. gingivalis* strain W83, that of ATCC 33277 has also been determined. Furthermore, the whole genome sequence of *P. gingivalis* with type II *fimA* will soon be revealed. Pan-genome analysis of *P. gingivalis* would be expected to clarify the differences of virulence among strains, and M-CGH could be utilized for analysis of the relationship between expression levels of microbial genes and periodontal situation. These future developments will be vital to identify the virulence/pathogenicity-related genes of *P. gingivalis*, while they will also be necessary for advancements in periodontal therapy and assessment of prognosis, by elucidating disease contributing clones of periodontal bacteria.

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Chapter 11
Molecular Typing of Nontuberculous Mycobacteria

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11.1 Introduction

The nontuberculous mycobacteria (NTM) encompass all members of the genus *Mycobacterium* other than the *Mycobacterium tuberculosis* complex and *Mycobacterium leprae*, the causative agents of tuberculosis and leprosy in humans and animals. The NTM are environmental bacteria that can readily be isolated from soil and both natural and man-made water systems [1]. Owing to the introduction of molecular tools for identification of NTM, the number of validly published species has risen to 135 in the year 2010. In the past two decades, the importance of NTM as causative agents of mostly opportunistic infections in humans has been increasingly recognized. In the 1950s, sporadic cases of tuberculosis-like pulmonary disease had been recorded, mostly in elderly male patients with a history of smoking or employment as miners [2]. In the 1980s, the HIV epidemic brought increased attention to NTM infections, particularly to the ones caused by *Mycobacterium avium*, as these were the most frequent opportunistic pathogens in patients with severe AIDS (i.e., CD4 cell counts <50 per nl), often causing disseminated infections. With the advent of highly active antiretroviral therapy, the incidence of NTM infections in HIV positive patients decreased strongly [3]. Overall, the incidence of

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disease caused by NTM seems to be increasing, mainly in areas where the incidence of tuberculosis is in decline [4, 5]. Pulmonary infections by NTM are now the most frequent human NTM infections, followed by lymph node infections in children [6]. Yet, as NTM disease is non-communicable infection, this is not a reportable condition in most countries, and exact epidemiological data is lacking. The background of the increased incidence remains poorly understood; in some regions it has been related to an increase in the number of aging patients at increased risk owing to the rising prevalence of chronic pulmonary diseases [5]. But also a higher degree of awareness, improved laboratory techniques and more frequent exposure to NTM contaminated shower water will presumably play a role in this. Despite the seemingly increasing incidence, the exact sources of infections in patients are rarely investigated by molecular typing and, if performed, typing rarely reveals an exact source of infection [1]. NTM (except *Mycobacterium ulcerans*) can be handled under biosafety class II conditions and can be examined in most microbiological laboratories. The important difference with typing *M. tuberculosis* lies more in the interpretation than in the practical performance of typing. As NTM disease does not spread from human to human, typing is generally performed to investigate possible sources of infection for individual patients, or to study the possibility of specimen contamination inside (e.g., cross-contamination of samples or contamination of laboratory appliances) or outside (e.g., contaminated endoscope washers or water sources) the microbiological laboratory [1, 6].

The still limited use of molecular typing tools in NTM implies that comparative studies of discriminatory power and the stability of the various molecular markers are still few and far between. Moreover, studies of discriminatory power of a single technique across various NTM species are notoriously lacking. Most studies focus on typing of a single NTM species and use a convenient sample. A complicating factor in typing of NTM is that, although the evolutionary time of divergence of NTM is thought to be much larger than for the *M. tuberculosis* complex, some (sub) species like for example *Mycobacterium malmoense* and *M. avium* subsp. *paratuberculosis* are genetically highly conserved and caution should be exerted in the interpretation of any typing result. Firm conclusions on epidemiological links suggested by finding isolates with identical or highly similar DNA fingerprint patters of such (sub) species should be avoided. More knowledge on the genetic population structure of NTM species is needed to overcome this problem. This issue will only be addressed after whole genome sequencing has been applied to large representative collections of NTM isolates from various sources. NTM infecting humans may vary and be a minor subset from the ones that are generally found in the environmental sources, like tap water [7], complicating the interpretation of typing results of isolates from both sources. Therefore, the general reliability of NTM typing can still be improved significantly. Nevertheless, in the last decade an increasing number of methods for molecular typing have emerged with different levels of application in the epidemiology of NTM. In this chapter we provide an overview of generic and species-specific molecular typing methods available for NTM, with examples of their use. Genetic diversity can be used for (sub)species identification and this requires semi-conserved genomic information. Strain typing for epidemiological research requires a higher level of polymorphism, but the degree of genetic variability is likely to differ strongly by (sub)species.

11.2 Generic Methods

11.2.1 Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) is a generic typing method that has been applied to typing of various NTM species. PFGE is performed by first embedding organisms in agarose (a "plug"), lysing the organisms in situ, and digesting the chromosomal DNA with restriction endonucleases that cleave infrequently. Thereafter, the agarose blocks yielding the chromosomal DNA fragments are inserted into the top of an agarose gel, and the restriction fragments are separated in the gel by alternating the direction of electric currents in a predetermined pattern enabling the movement of very large DNA fragments. The resulting banding patterns can be compared, using a predefined set of criteria for strain relatedness [8]. Minor adaptations have been made to apply this method to species including *Mycobacterium abscessus* [9], *Mycobacterium haemophilum* [10], and *M. malmoense* [11]. In general, this method is technically demanding, as it involves the inclusion of intact, non-aggregated bacterial cells with high molecular weight DNA into the agarose blocks, before additional handling. Therefore, an important drawback to PFGE typing is the occurrence of DNA degradation, for instance described for a specific clone of *M. abscessus* [9]. This notorious pathogen that particularly affects patients with Cystic Fibrosis [12] is a frequent clinical isolate in many parts of the world [6], which emphasizes the need for a robust typing technique. The use of thiourea containing buffer solutions has been shown to decrease DNA degradation in *M. abscessus* [9].

For *M. abscessus*, PFGE typing has been used to detect laboratory cross-contamination events [13], to investigate the clonality of strains from patients with wound infections after surgery in Brazil [14] and to exclude human transmission among patients with cystic fibrosis [12]. Inter-human transmission of *M. malmoense* infection in an area of Scotland with an unexpectedly high incidence rate could also be refuted after PFGE typing revealed strain diversity [11]. Lastly, PFGE was instrumental in pointing out nail salon footbaths as the source of an outbreak of *Mycobacterium fortuitum* skin infections in California [15].

11.2.2 Amplified Fragment Length Polymorphism Typing

Amplified fragment length polymorphism (AFLP) is a method in which DNA is isolated and digested by one or two restriction enzymes (generally one with an average and one with a high cutting frequency) and restriction fragments are ligated with double stranded oligonucleotide adapters. Using labeled primers based on the adapters, the restriction fragments are amplified in a stringent PCR. The 40–200 products are separated on agarose gels to produce the fingerprint pattern [16].

This technique has been successfully applied to various NTM species including the *M. avium* complex, *M. haemophilum* [17] and *Mycobacterium marinum* and *M. ulcerans* [18]. AFLP analysis also clearly distinguishes between the latter two species, conforming that although these species are difficult to distinguish by conventional methods, they are genetically significantly different.

11.2.3 Random Amplified Polymorphic DNA Typing

Random amplified polymorphic DNA (RAPD) is easy to perform, and based on the amplifications of DNA fragments using short, random primers that fit frequently present repetitive sequences. Amplicons are labeled for visualization and separated on basis of molecular weight on agarose gels. This method has several limitations, of which the apparent lacks of inter-test and inter-laboratory reproducibility are the most pressing. In one small-scale comparative study of RAPD and PGFE typing for *M. fortuitum* isolates, the discriminatory power of RAPD was lower than that of PFGE [19]. Nonetheless, this technique has been used to type isolates of numerous NTM species, including *M. abscessus* [20], *Mycobacterium phocaicum* [21], *Mycobacterium gordonae* [22], and *Mycobacterium szulgai* [23]. RAPD typing has been particularly well described for *M. abscessus*, as this species is often difficult to type by PFGE, owing to DNA degradation [9]. Typing was performed to examine possible laboratory cross-contamination with *M. abscessus* [20, 24], (pseudo)outbreaks of *M. gordonae* related to contaminated bronchoscopes and washers [22], as well as the diversity of *M. szulgai* isolates in a retrospective survey in the Netherlands [23].

11.2.4 Repetitive Sequence (rep)-PCR

Rep-PCR typing has been used in many genera, including the genus *Mycobacterium*. It applies primers based on repetitive elements in bacterial or fungal genomes to amplify regions between these repetitive elements; the number and size of these regions are visualized on agarose gels or in microfluidics chips [25]. This technique has been successfully applied in typing of *M. abscessus* group bacteria [26] and *M. avium* [27]; for *M. avium* typing, its discriminatory power has been suggested to equal that of IS*1245* restriction fragment length polymorphism typing, the current gold standard [27]. Inter-test reproducibility for this test remains to be established.

11.2.5 Multi Locus Sequence Typing

The use of Multi locus sequence typing (MLST) in NTM is of recent date. Its use is based on the recognition that within NTM species, a limited degree of genetic diversity is reflected in housekeeping genes. Thus, if sequence data of multiple semi-conserved

genes were combined, a fair degree of resolution could be obtained. The housekeeping genes most frequently used for this purpose include the 65 kDa heat shock protein (*hsp65*), RNA polymerase beta subunit (*rpoB*), superoxide dismutase (*sodA*) gene and the 16S-23S internal transcribed spacer. Combinations of up to ten genes have improved our understanding of the intra-species genetic divergence and evolution of NTM [28], but have also proven to be valuable in investigations of laboratory outbreak settings [21]. An example of this use is an outbreak of NTM bacteremia in a hospital in Texas, where sequence analysis of multiple housekeeping genes was combined with generic typing methods to reveal that cases were unrelated and in one case could be linked to a near-patient water source [21].

11.3 Species Specific Methods

11.3.1 Restriction Fragment Length Polymorphism Typing

Restriction fragment length polymorphism (RFLP) typing is a method in which DNA is extracted and purified from cultured mycobacteria and digested with restriction enzymes. The restriction fragments are subsequently separated on an agarose gel; separated restriction fragments are transferred to a DNA membrane. Repetitive genetic elements present in the various restriction fragments, mostly insertion sequences, can then be visualized by adding a peroxidase-labeled probe with a DNA sequence complementary to element's DNA sequence to a hybridization buffer, which is poured onto the membrane. The restriction fragments that the probe hybridizes to are highlighted by adding substrates that produce a chemiluminescence reaction or a dye. The RFLP patterns are visualized by putting a light-sensitive film on the packed membrane in a light-blocked cassette [29]. Various repetitive elements have been exploited in typing of isolates of various NTM species. The most frequently used are the closely related insertion sequences IS*1245* and IS*1311*, which are present in 1–30 copies in different subtypes of the *M. avium* complex. On basis of the number and position of bands in the IS*1245* or IS*1311* RFLP patterns, various clades of *M. avium* and its subspecies can be distinguished that are related to infections in birds, pigs, humans and ruminants [30–32]. Of note, this element has been demonstrated in the genomes of a few NTM species other than *M. avium*; its applicability to type strains of these species (*Mycobacterium nonchromogenicum*, *Mycobacterium scrofulaceum*, *M. malmoense*) remains to be investigated [33]. Other insertion elements that are known and haven been used for typing of *M. avium* complex bacteria include IS*900* present in *M. avium* subsp. *paratuberculosis* [34], IS*901* present in *M. avium* subsp. *avium* [35], IS*902* present in *M. avium* subsp. *silvaticum* [36] and IS*666*, IS*1110* and IS*1626*, whose distribution among *M. avium* strains has been less well studied [37–39]. IS*900*, IS*901*, IS*902*, and IS*1245* can be used for the identification of the various *M. avium* subspecies, as well as for typing purposes within these subspecies. One of the most important conclusions in IS*1245* RFLP typing was that birds are infected by a genetically highly conserved type of

M. avium strains invariably revealing the same three-band pattern, while the banding patterns of *M. avium* isolates from porcine and human sources revealed highly variable and multi-banded patterns. On the basis of these observations and to serve the clarity in the epidemiology of *M. avium* infections, it was proposed to reserve the naming *M. avium-avium* for the bird-type isolates and to introduce the designation *M. avium hominissuis* for the typical isolates from humans and pigs [31].

Outside the *M. avium* complex, RFLP typing has been less pursued, but potentially useful insertion sequences have been described in a variety of species; these include IS*1407* in *Mycobacterium celatum* [40], IS*1081* and IS*1395* in *M. xenopi* [41, 42], IS*1511*/*1512* in *M. gordonae* [43], IS*2404* in *M. ulcerans*, IS*2606* in *M. ulcerans* and *Mycobacterium lentiflavum* [44], IS*1652* in *Mycobacterium kansasii* [45], and IS*6120* in *Mycobacterium smegmatis* [46]. Genetic elements other than insertion sequences have also been explored in RFLP typing. An important example is the major polymorphic tandem repeat (MPTR) element that has been mainly applied in typing studies of *M. kansasii*, though is also present in *M. gordonae* [47]. For *M. kansasii*, this technique has revealed the existence of multiple subtypes within this species [45]; these subtypes have later been shown to have different degrees of pathogenicity in humans [48], which proved the value and utility of genotyping for this species. Of note, one RFLP target, the (GTG)5 oligonucleotide has been used across a wide array of species [49]. Yet, this potentially generic NTM typing technique has not been widely applied after its formal description.

11.3.2 Variable Number of Tandem Repeats Typing

Variable number of tandem repeats (VNTR) typing is a very recent addition to molecular typing tools for nontuberculous mycobacteria and has so far only been applied to *M. avium* and *Mycobacterium intracellulare*, as these is the most commonly isolated NTM species worldwide [32, 50]. The principle of VNTR typing is that by PCR, particular genomic regions with previously characterized tandem repeats are amplified. The size of the region, minus the sizes of the flanking regions and divided by the length of the tandem repeat provides the investigator with the number of repeats present in the locus. By measuring the number of repeats present in a predefined number of loci in the genome, a multi-digit numerical code is obtained, that functions as the fingerprint [51]. Advantages of this method are that it is PCR based and thus needs only a limited amount of DNA. This prevents the 2–6 weeks culture delay that makes many typing techniques for mycobacteria far less than real-time. The output format, a numerical code, allows easy comparisons and data exchange between laboratories. Similar VNTR methods have earlier been developed for typing of *M. tuberculosis* complex bacteria and have become the gold standard [52]. The discriminatory power observed in *M. avium* and *M. intracellulare* are also considered promising [32, 50].

11.4 A Special Case: *M. ulcerans*

M. ulcerans is the causative agent of a severe, debilitating skin infection known as Buruli Ulcer Disease (or, previously, Bairnsdale Ulcer). Owing to its pathogenicity, *M. ulcerans* stands out among the NTM, and is therefore considering to be a group in itself and a biosafety class III pathogen, hence its special mention in this chapter. The disease was first described in Australia, but is most common in (sub)tropical areas in West-Africa, Latin America and to a lesser extent in East Asia [53]. It causes nodular lesions, which can progress to extensive ulcers. Treatment is either by prolonged courses of antibiotics, especially effective in the early nodular stage, or by surgery in the ulcerative phase. Its causative agent, *M. ulcerans*, is difficult to culture in vitro and this has for long prevented molecular typing. By now, multiple potential targets for typing have been established. The first was the IS*2404* element, which is present in >50 copies in its genome; RFLP typing using this target has helped to define geographical spread of particular lineages of *M. ulcerans* [18]. Recently, 13 VNTR loci have been described and applied to confirm clonal relationships between patient and environmental isolates of *M. ulcerans* [54]. Typing may help to determine the exact sources and modes of transmission of this infection. Waterborne insects have been shown to carry *M. ulcerans* [53], and typing may help to establish the possible role of this vector in disease transmission.

11.5 Gold Standards and Future Prospects

Given the increase in incidence and prevalence of human disease caused by NTM, the demand for molecular typing to address its etiology is likely to increase. Although generic methods are available, their discriminatory power in NTM has not been well studied, as many are in fact methods applied to *M. tuberculosis* complex bacteria that happened to work for specific NTM species as well. Moreover, the level of genetic diversity in NTM (sub)species is not well examined, and this hampers the interpretation of typing results. PFGE typing has at least been widely applied in various NTM species, with minor species-specific modifications. Owing to its wide use, it could be considered the gold standard for species other than *M. avium*. For *M. avium*, IS*1245* RFLP typing is the widely recognized reference method [30]. VNTR typing has methodological and practical advantages over RFLP, mainly in its turnaround time; this PCR-based typing method requires fewer bacteria, thus shortening culture delay. Its discriminatory power, however, remains to be fully established. MLST also has the potential to become a reference method, although it current use is still limited to reference laboratories with full access to sequencing facilities. The use of whole genome sequencing in NTM has been remarkable slow [55]; its use in *M. tuberculosis* is now rapidly increasing, including its use in the molecular epidemiology [56]. Once whole genome sequencing has become more generally available it is likely this approach will be the ultimate typing

tool, yielding genetic information on the (sub) species identity, the broad and fine phylogenetic branching and variation on strain level. This will serve the research on the etiology, host-range, transmission, clinical relevance of NTM, and even treatment of the disease it causes. Noting the significant decreases in the price of sequencing, this new era will be entered soon.

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Chapter 12
Molecular Typing of *Neisseria meningitidis*

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12.1 Introduction

Neisseria meningitidis (Nm) is an exclusive human pathogen that is responsible for life-threatening invasive infections. Nm portal of entry is the nasopharynx and asymptomatic carriage, ranging from 10 to 35% in young adults [1], is the most frequent output of interaction of Nm with its unique host. Nm transmission is direct through airborne salivary droplets during person-to person contacts. However, particular virulent strains are able to provoke invasive meningococcal infections (IMI) such as septicemia and meningitis. Meningitis occurs when Nm reach the meninges to provoke inflammation that mainly involves the leptomeninges (the arachnoid and the pia mater). In general, bacteria gain access to the meninges through spread from blood and after crossing the blood brain barrier.

IMIs occur as sporadic cases (in Europe and North America) with occasional outbreaks. Annual incidence in Europe varies between 0.31 and 4.92 per 100,000 per year [2]. However, epidemics occur in developing countries and particularly in the African countries of meningitis belt. IMI notification is mandatory. Efficient management requires a rapid and reliable identification for etiological diagnosis and specific measures should be undertaken for the immediate management of IMI and for global epidemiological surveillance:

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– *At the patient level*. Prompt adequate antibiotic therapy is crucial for good prognosis. Rapid etiologic diagnosis and determining the susceptibility are crucial.
– *At the level of contact persons*. Rapid identification of serogroup is important to implement preventive measures among contact persons (vaccination and/or chemoprophylaxis). To prevent potential epidemic spread. Indeed, 12 serogroups based on the immune specificity of the meningococcal capsule are currently known. Five of these serogroups (A, B, C, Y and W-135) are most frequently encountered in invasive infections. However, polysaccharide-based vaccines are only available against isolates of serogroups A, C, Y and W-135
– *At the community level*. Typing and tracking the emergence and/or the spread of meningococcal isolates are essential elements at the global level in the surveillance of IMI.

12.2 Classical Methods and Their Limitation in Identification and Typing of Meningococcal Isolates

Typing of meningococcal isolates seems hence essential and need to address critical issues in the management of invasive meningococcal infections. Culture isolation of meningococcal strains allows addressing these points.

– Antibiotic susceptibility. Conventional antibiogram is recommended to be performed using E-test on Mueller-Hinton medium supplemented with sheep blood [3] for the antibiotics that are currently used in treatment (mainly beta-lactam antibiotics and chloramphenicol in low resource countries) and chemoprophylaxis (rifampicin, ciprofloxacin, and ceftriaxone). It is important to underline that a thorough knowledge on meningococcal susceptibility to antibiotic is needed even for presumptive early antibiotic therapy. Indeed, presumptive treatment requires extensive and reliable data on the epidemiology and the antibiotic resistance of Nm [4]. However, the absence of cultured bacteria precludes this analysis. Other problems of the antibiogram are the lack of consensus on breakpoints (minimal inhibitory concentrations, MIC, that allow classification of isolates into susceptible, intermediate or resistant) and the suboptimal correlation between laboratories in determining MICs. In an interlaboratory study, agreement among laboratories in determining MICs was 87.6% for penicillin G and 78.8% for rifampicin [3].
– Serogrouping is usually performed by agglutination and latex agglutination reactions using specific sera on cultured colonies or directly on clinical samples such as blood, cerebrospinal fluid (CSF) and urine. However, cultured bacteria may lack due to early antibiotic therapy that is highly recommended when IMI is suspected [5]. Indeed, CSF sterilization is rapidly obtained (4 h) after the onset of the treatments [6]. Moreover, cross reaction may be encountered with several other bacterial species [7].
– Typing of isolates. Conventional phenotyping of Nm relies on serological tests. Serogrouping but also serotyping and serosubtyping are usually used. These tests

employ specific monoclonal antibodies based on the immune specificity of the class 2 or class 3 outer membrane proteins, PorB (serotype), and the class 1 outer membrane protein, PorA (serosubtype). Absence of cultured bacteria is also a major drawback in phenotyping of Nm isolates. Moreover, increasing numbers of isolates remain not-typeable due to lack of adequate monoclonal antibodies. Indeed, meningococcal isolates undergo frequent horizontal DNA exchanges through transformation and recombination as Nm is naturally competent for transformation. Meningococcal isolates are hence highly diverse. Conventional methods of typing are now problematic due to failure to culture bacteria. Nm fragility and early antibiotic treatment potentiate this failure that may encountered among 58–96% of cases [5]. Lack of adequate antibodies for typing is another major problem. Moreover, the outer membrane porins (PorA and PorB) are subject to a strong selection by the host immune response. Phenotyping may not reliably inform on the genetic relatedness between meningococcal isolates. Phenotyping methods alone for isolates characterization are then inadequate for epidemiological analysis [8, 9]. Molecular methods have been recently developed to provide more reliable typing of meningococcal isolates and to overcome limitations of conventional typing methods:

- Culture negative cases of IMI can hence be explored and the corresponding isolates typed.
- Non-typeable isolates can be easily characterized.
- Genetic relationships among isolates are directly addressed.

12.3 Molecular Characterization Methods That are Needed for the Immediate Management of IMI

12.3.1 Identification of N. meningitidis

This chapter mainly deals with molecular typing and does not focus on molecular diagnosis methods of cultured bacteria. A brief description of non-culture methods of identification of Nm is mentioned. DNA-based methods have been used for the identification of many pathogenic bacteria incriminated in infections of central nervous system [8]. Amplification of DNA using PCR approaches is usually used. These methods most frequently employ PCR on meningococcal specific genes such as *ctrA* gene that encodes an outer membrane protein involved in capsule transport, *crgA* gene that encodes a transcriptional regulator belonging to the LysR family, the major porin encoding gene (*porA*) and the multi-copy insertion sequences (IS*1106*). Their performance was compared in an interlaboratory study with a mean sensitivity and specificity of 89.7% and 92.7%, respectively among the participating laboratories [10].

Broad range amplification of 16S rRNA encoding gene is another alternative. A second PCR or sequencing of the first PCR may then allow identifying the bacterial species [11]. This method permits the identification of several bacterial species involved in meningitis with variable sensitivity (the detection level for bacteria was

10^3 CFU/mL for *N. meningitidis*) [11]. However, contamination with exogenous DNA is problematic [12]. Diagnostic PCRs of Nm should therefore be submitted to general quality assurance schemes that address several issues (see ref. [13] for a review):

- The issues of samples, their storage, sending and DNA extraction.
- PCR laboratory environment, personnel training, equipment and validation of protocols. Indeed, Diagnostic PCR should be performed in a "PCR module" that should be separated for other activities and modules in the laboratory. In general, the "PCR laboratory" should contain three separate modules organized sequentially with a "one way" workflow and gradient of atmospheric pressure.
- External QA interlaboratory studies are essential upon validation of PCR protocols.

It is interesting to note that PCR methods were also applied efficiently in low resource countries such as Niger, Burkina Faso and Sudan [14, 15]. Indeed, culture may be difficult to implant in remote areas. PCR methods may offer a reliable logistic choice.

12.3.2 Genogrouping

Serogroup distribution is an important information in the management of IMI and, particularly, in vaccination strategies as currently available vaccines are based on capsular polysaccharides (A, C, Y, and W-135). PCR-based molecular approaches have been used to perform genogrouping (prediction of the meningococcal serogroup). These approaches can be applied on cultured bacteria and as non-culture methods after a positive PCR of identification of *N. meningitidis* (see the Sect. 12.3.1 above). Genogrouping PCRs have been described for isolates belonging to serogroups A, B, C, Y, and W-135, X, Z, and 29E of *N. meningitidis* [16–23]. All these PCR methods target genes that are responsible for the specificity of the corresponding serogroup. *siaD* gene encoding the enzyme that is involved in polymerization of the sialic acid units in sialic-acid containing capsules (serogroup B, C, Y, and W135) can be used to predict these serogroups. The *mynB/sacC* gene can be targeted to predict serogroup A. The *xcbA* is most likely the putative capsule polymerase and can be used to predict serogroup X [24]. For genogrouping of isolates of the serogroups 29E and Z, PCR-based assays were reported that target specific regions of the *ctrA* gene [21]. PCR of genogrouping was found to be less sensitive than that of Nm identification. In an interlaboratory study, the performance of genogrouping was variable between participant laboratories with a mean sensitivity of 72.7% [10]. PCR-based techniques enhanced by 44% the ascertainment and genogrouping of IMI [25].

12.3.3 Molecular Typing of Meningococcal Antibiotic Susceptibility/Resistance

Molecular typing of antibiotic susceptibility may overcome technical difficulties of the antibiogram and allow prediction of antibiotic susceptibility even in culture negative cases. Molecular methods should be based on our understanding of mechanisms

antibiotic resistance. Molecular detection of genetic events associated with bacterial resistance to antibiotics has been described for several antibiotics that are currently used in treatment and prophylaxis of IMIs.

12.3.3.1 Molecular Typing of Susceptibility to Beta Lactams

Alterations of penicillin binding protein 2 (PBP2) are associated with reduced susceptibility of *N. meningitidis* to penicillin G [26]. This phenotype is hence not conferred by beta-lactamase enzymes and has evolved under positive selection for alterations in *penA* gene encoding PBP2. Alterations in the C-terminal part of PBP2 (amino acids 298–581) are directly linked to reduced susceptibility to penicillin G of *N. meningitidis*. Strains with reduced susceptibility to penicillin G (PenI) showed 8.5–14.4% sequence divergence [27] in this region of PBP2. Five positions are always altered in PenI isolates and correspond to codons F504, A510, I515, H541, and I566 [28]. These positions are located around the conserved KTG (lysine, threonine, and glycine) motif that is most likely part of the structure that forms the active site of PBP2 [27, 29]. Sequencing of *penA* allowed detecting of these alterations and hence permitted the prediction of PenI phenotype [28]. Other PCR-based methods use restriction polymorphisms or real time PCR to detect to alteration of *penA* [30]. These methods can be applied on cultured bacteria but also directly on clinical samples (blood or CSF). *penA* typing also helps in general molecular typing scheme of meningococcal isolates as it may discriminate between closely related isolates.

Molecular methods can help to better define breakpoints for penicillin G. Indeed, *penA* sequencing allow determining highly related alleles of *penA* (≥99% identity) with no modification of the above mentioned five critical positions. Geometric mean of MICs of penicillin G for isolates harboring these alleles ranged between 0.055 and 0.094 mg/L. Susceptible isolates (PenS) were hence defined using the breakpoint MIC ≤ 0.094 mg/L [28]. At the opposite, highly altered *penA* alleles harbored at least the five critical positions modified. The geometric mean of MIC of penicillin G for the corresponding meningococcal isolates ranged between 0.112 and 0.511 mg/L. Penicillin non susceptible (intermediate PenI isolates) were hence defined using the breakpoint MIC > 0.094 mg/L [28]. These data are available on a specific Web site for *penA* typing (http://pubmpst.org/neisseria).

12.3.3.2 Molecular Typing of Susceptibility to Rifampicin

Rifampicin is an antibiotic of choice for chemoprophylaxis of meningococcal disease. Isolates with high resistance level to rifampicin (MIC>32 mg/L) seem to be rare among clinical isolates and are directly correlated to mutations at (or close to) the codon corresponding to H552 in *rpoB* gene encoding the beta subunit

of the RNA polymerase [31]. Sequencing of the corresponding fragment of *rpoB* allows predicting rifampicin resistance using both cultured bacteria and clinical samples [31, 32].

12.3.3.3 Molecular Typing of Susceptibility to Chloramphenicol

Chloramphenicol (the oily form) is still an antibiotic of choice in the management of IMI in countries within the African meningitis belt [33]. Meningococcal isolates showing high-levels of resistance to chloramphenicol has been reported in Vietnam, France and Australia. They emerged through the acquisition of the *catP* gene encoding chloramphenicol acetyl transferase that inactivates chloramphenicol [34, 35]. Molecular typing by PCR amplification and/or molecular hybridization permits a direct detection of *catP* gene. Such a method was used to show that no *catP* positive isolates were so far detected in Africa [36].

12.3.3.4 Molecular Typing of Susceptibility to Ciprofloxacin

Ciprofloxacin is another antibiotic of choice for chemoprophylaxis of meningococcal disease. Rare ciprofloxacin resistant isolates were reported in Argentina, Australia, France, India, Israel, Spain, and USA. This resistance is linked to mutations at the codon 91 and/or 95 of the gene encoding subunit A of DNA gyrase (*gyrA*) [37–43] that can be detected by PCR amplification and sequencing [39, 40, 43].

12.4 Molecular Typing Methods for Epidemiological Surveillance

The need for discriminatory and reliable techniques for tracking isolates of *N. meningitidis* has driven the development of powerful approaches of typing. Due to the high rate of DNA horizontal exchanges among meningococcal isolates, it is mandatory for a reliable analysis to target several genetic loci in each isolate. The Multilocus enzyme electrophoresis (MLEE) allowed for the first time to show the high diversity of meningococcal isolates and the existence of particular genetic lineages that are associated with disease [44]. This method discriminates the isolates on the basis of differences of the electrophoretic mobility of isoenzymes encoded by the different alleles of a given gene. Several enzymes are usually analyzed simultaneously and their electrophoretic profile is called electrophoretic type (ET). Closely related ETs can be clustered together in one clonal grouping (clonal complex). However, this method is time consuming and requires cultured bacteria. The results are not portable among laboratories and the method requires the use of reference strains. The development of high-throughput sequencing approaches allowed the development of molecular new methods of typing.

12.4.1 Multilocus Sequence Typing MLST

This method analyzes the polymorphism (at the level of nucleotide sequence) of seven chromosomal genes in *N. meningitidis* that encode metabolic enzymes (*abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC*, and *pgm*). DNA sequencing is employed to determine the nucleotide sequence of approximately 450-bp from PCR products of these genes. For each gene an allele is then defined on the basis of at least one nucleotide difference. The combination of the seven corresponding alleles of these seven tested genes defines the sequence type (ST) of a given isolate. Close STs are clustered into clonal complexes [45]. This method does not require the use of reference strains. It is portable and results are easy to compare between different laboratories. This comparison is facilitated by a Web site for this approach (http://pubmpst.org/neisseria). This method can also be used as a non-culture method and hence be applied directly for clinical samples (CSF and blood) [46]. Safe exchange of isolates between laboratories is also facilitated as bacterial extracts can be sent to reference laboratories for sequencing instead of viable bacteria. MLST is now the gold standard method not only in typing isolates of *N. meningitidis* but also in typing of several other bacterial species [47]. An extensive amount of information is now available from MLST analysis that improved our view on population genetics and global molecular epidemiology of *N. meningitidis*. It permits a real-time view of the currently circulating isolates worldwide. Few major clonal complexes that are involved currently in most cases of IMI are identified such as isolates belonging to the clonal complex ST-5 (serogroup A), the clonal complex ST-11 (mostly serogroup C but also serogroups W-135 and B), the clonal complex ST-8 (mostly serogroup C but also serogroup B), the clonal complex ST-32 (mostly serogroup B), the clonal complex ST-41/44 (mostly serogroup B) and the clonal complex ST-269 (mostly serogroup B) [48]. It is noteworthy that correlation of genotyping data with those of conventional phenotyping that relies on serological tests (Serogrouping serotyping and serosubtyping) is not complete.

12.4.2 porA Sequencing

Conventionally, serosubtypes are determined by serological testing using monoclonal antibodies that recognize epitopes in three variable regions (VR1, VR2 and VR3) located in the outer membrane protein PorA. Non-serosubtypeable strains are frequent due to lack of corresponding monoclonal antibodies. Serosubtyping also overlooks slight variation within a given variable region that may not change epitope recognition of this variable region by the corresponding monoclonal antibody. Genosubtyping of *N. meningitidis* can be performed by amplification of *porA* gene and sequencing the variable regions encoding VR1, VR2, and VR3 of the *PorAx* that then allows prediction of serosubtypes through the Web site neisseria.org. This method can be applied on cultured bacteria and directly on

clinical samples. Non-serosubtypeable strains are hence easily characterized. Moreover, minor variations that are overlooked by serosubtyping could be detected by DNA sequencing [49–52]. However, isolates that are deleted for *porA* exist. Indeed, outbreak of meningococcal disease caused by PorA-deficient *N. meningitidis* is described [53]. In addition, sequencing of *porA* can not inform on the expression of this gene and the production of PorA at the bacterial surface. This information is of importance as PorA is targeted by bactericidal antibodies generated by outer membrane vesicle (OMV)-type vaccine [54].

12.4.3 fetA Sequencing

fetA gene (also called *frpB*) encodes an iron-regulated siderophore receptor that is present in the majority of meningococcal isolates. This protein is immunogenic and contains several polymorphic site that may be used in typing of meningococcal isolates [55]. *fetA* typing targets a particular variable region on the gene that is amplified by PCR using primers proposed at the curated database on the Web site neisseria.org. This method can be applied on cultured bacteria and directly on clinical samples. Sequence typing of this variable region allows identifying FetA types that when combined to *porA* sequencing and MLST analysis allow high discrimination level among meningococcal isolates [56].

12.4.4 Pulsed Field gel Electrophoresis

This method is based on the analysis of macro-restriction profiles of genomic DNA. However, it requires viable bacteria in order to prepare and purify enough genomic DNA for this analysis. It is a sensitive method for analyzing genetic relatedness and diversity among isolates. Indeed, it allowed a fine discrimination among isolates of serogoup W-135 belonging to the clonal complex ST-11 upon the expansion in the year 2000 of a particular clone within this lineage that was linked to the pilgrimage to Mecca [57–59]. This technique may be combined with genomic hybridizations using specific DNA probes to study genetic arrangement of the corresponding chromosomal loci [60]. Capsule switching events from serogroup C to serogroup B were explored using pulsed field gel electrophoresis (PFGE) analysis followed by a Southern hybridization using *siaD* probes specific for serogroup B or serogroup C [61]. PFGE is a highly discriminatory method and is well adapted for local (short-term) epidemiological analysis to establish a possible clonal nature of an outbreak. However, comparison of results between different laboratories is still difficult and needs better standardization of protocols.

12.4.5 Variable Number Tandem Repeats

Short DNA tandem repeats have been reported in coding or promoter regions of genes in Nm. Their numbers may vary during DNA replication through slipped-strand mispairing [62]. PCR-based determining of the number of the variable number tandem repeats (VNTR) was developed [63] and used as a simple and reliable method of molecular typing. As for PFGE, VNTR is well adapted to explore the relatedness among isolates during outbreaks (short-term epidemiology). A good correlation with the epidemiological data was achieved when VNTR was used to analyze two outbreaks in Greece [64]. Moreover, this method can be directly used on clinical samples as a non-culture typing method [65].

12.4.6 Sequencing of Other Meningococcal Genes

Efforts are continuing by several reference laboratories and in particular by members of the European Monitoring Group for Meningococci, the EMGM, (now the European Meningococcal Disease Society) to explore new targets for more extensive molecular typing of meningococcal isolates. Efforts are focusing on genes encoding components of the recombinant meningococcal vaccines that are under development. In particular, the meningococcal factor H binding protein (fHbp) is a major component of these recombinant vaccines. Meningococcal Fhbp binds factor H, a key regulator of the alternative complement pathway. Recruitment of factor H to the surface of the bacterium may inhibit complement activation and hence enabling Nm to evade complement-mediated lysis [66, 67]. Polymorphism of *fhbp* has then major impacts on the potential use of these recombinant vaccines [68]. PCR amplification, sequencing, and molecular typing of *fhbp* are being established on the Web site neisseria.org. The importance of studying diversity and the expression of fHbp is crucial to determine the strain coverage by the recombinant vaccines.

12.5 Conclusions

The evolution of *N. meningitidis* occurs mainly through DNA horizontal DNA exchanges. The emergence of new genotypes and/or the diversification of old genotypes are major traits in meningococcal evolution with the potential epidemic expansion of the new variants. The need of reliable molecular typing methods is highly warranted that should keep up-to-date our surveillance of the currently circulating isolates. Typing of meningococcal isolates is now moving towards the widespread use of standardized molecular typing protocols. Molecular typing methods should be employed:

– To improve the immediate management of IMI: treatment of the patient, application of preventive masseurs (methods for genogrouping and molecular typing of resistance to antibiotics) and outbreak detection (short-term epidemiology)
– To improve our understanding of the global epidemiology of IMI and in particular using methods such as MLST.

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Chapter 13
Molecular Typing in Bacterial Infections Haemophilus spp.

Marina Cerquetti

13.1 Introduction

The genus *Haemophilus* includes several species associated with humans, but the major pathogen undoubtedly is *Haemophilus influenzae*. For a detailed description of the different *Haemophilus* species, numerous microbiological texts are available. This chapter is a brief presentation of *H. influenzae* disease in view of the major issues raised in the Hib post-vaccine era and a review of the current molecular methods for both diagnosis and typing of this important human pathogen.

13.1.1 H. influenzae Disease in the Hib Post-Vaccine Era

H. influenzae is a pleomorphic Gram-negative bacterium belonging to the family of *Pasteurellaceae* that colonizes the upper respiratory tract of healthy humans [1]. However, this organism is responsible for a wide variety of diseases ranging from respiratory tract infections to potentially life-threatening diseases such as meningitis, epiglottitis, and septicemia [2]. Isolates of *H. influenzae* can be segregated into encapsulated and nonencapsulated forms. Encapsulated *H. influenzae* expresses one of six structurally and antigenically distinct polysaccharide capsules, designated from a to f [3]. Strains lacking capsule are defined by their inability to react with antisera against the recognized polysaccharide capsules and are referred to as nontypeable.

Pathogenicity of typable or nontypeable *H. influenzae* (NTHi) is partially different. The capsule is the major virulence determinant of encapsulated *H. influenzae* [4]. It is well known that polysaccharide capsules mediate resistance to host defense

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mechanisms, including phagocytosis and complement-mediated killing. Encapsulated strains of *H. influenzae* are an important cause of invasive disease such as meningitis, sepsis, epiglottitis, and septic arthritis. Before vaccination against type b capsular polysaccharide was introduced in the late 1980s, *H. influenzae* type b (Hib) was the leading cause of meningitis and invasive disease in infants and young children, worldwide [5]. Other serotypes are less common, but small outbreaks and sporadic cases have been reported. The routine immunization of infants with conjugated Hib vaccines has dramatically reduced the incidence of invasive Hib disease in developed countries [6, 7]. However, concern about the potential emergence of non-vaccine preventable strains (both encapsulated non-b and NTHi strains) as important pathogens has arisen, although, so far, no robust evidence of serotype replacement has been obtained following large-scale Hib immunization [8–13]. Nevertheless, focus of investigations has moved from Hib to encapsulated non-type b cases and several studies from different countries have reported careful phenotypic and genotypic characterization of type a, e and f *H. influenzae* isolates causing invasive disease [14–17]. Besides the issue of serotype replacement, it is important to mention what recently occurred in the UK, where, despite the initial marked success of the Hib vaccination program, a resurgence of invasive Hib disease cases was observed in all age classes including fully vaccinated children, 8 years after vaccine introduction [18, 19]. Although a rapid decline in Hib cases was obtained following the implementation of a Hib booster campaign [20], the UK experience emphasizes the value of long-term monitoring of immunization programs through laboratory-based surveillances that are able to differentiate vaccine-preventable cases from other cases.

In encapsulated *H. influenzae*, molecular research mainly focused on polysaccharide capsule genes. In each serotype, the genes involved in the production of the capsules are organized within a capsulation (*cap*) locus, which contains three functionally distinct regions, I, II and III [21]. Regions I and III are common to all capsular types and contain genes necessary for transport and process of the capsular material, while region II contains serotype-specific biosynthesis genes [22, 23]. In most invasive Hib isolates, the *cap*b locus lies between direct repeats of the *IS1016* insertion element and contains a duplication of the genes carrying a 1.2-kb deletion at the 5′ end of the *cap* repeat within the *IS1016* element and the *bex*A gene (necessary for polysaccharide export) [21, 24]. Such a deletion has been supposed to stabilize the duplication, resulting in increased capsule production and virulence [25]. Direct repeats of the *IS1016* have also been suggested to provide a molecular substrate for further *cap* gene sequence amplification [21]. Clinical Hib isolates harboring more than two copies of the *cap*b locus (multiple-copy strains) have been observed [26, 27]. In serotypes non-type b strains, the *IS1016* insertion element either flanks or does not flank the *cap* locus, depending on the serotype. If *IS1016* is physically associated with the *cap* locus, the latter can be amplified.

NTHi is an important cause of human respiratory tract infections [28]. In particular, this organism is a responsible for 20–30% of all cases of acute otitis media in infants and children and possibly a higher percentage of recurrent episodes [29]. In addition, NTHi is a common cause of sinusitis and community-acquired pneumonia and it is frequently implicated in acute exacerbation of chronic obstructive pulmonary

disease as well as in cystic fibrosis [30]. Besides, NTHi may be responsible for invasive disease (meningitis and sepsis) among adults, especially in patients with underlying predisposing conditions such as advanced age, immunosuppression and chronic lung disease [31]. Rarely, invasive NTHi disease may occur in infants and children, where it presents predominantly with septicaemia and pneumonia [32, 33], but also with meningitis, even in children without underlying predisposing conditions [34, 35]. As far as the issue of "replacement disease" is concerned, according to several population-based studies, so far, no increase in frequency of cases of NTHi meningitis in children has been observed, following the introduction of Hib conjugate vaccine, although NTHi has been found to be the predominant serotype [35–37]. Dworkin et al. described a significant increase in the incidence of invasive NTHi disease in people aged ≥65 years in Illinois, USA [38].

The initial step in the pathogenesis of NTHi disease involves establishment of bacteria on the rhino-pharyngeal respiratory mucosa followed by contiguous spread within the respiratory tract and, occasionally, to sterile sites. Several surface structures have been reported to affect virulence, including lipooligosaccharide, a major component of the *H. influenzae* cell wall, and many surface-associated proteins such as HMW1 and HMW2 proteins, Hia, Hap, hemagglutinating pili, and P5 outer membrane protein, but there is no single feature common to of all disease-associated strains [39, 40]. After establishment on the mucosal surface, successful persistence of bacteria requires evasion of host defense. NTHi achieves this objective both by producing an extracellular endopeptidase-called IgA1protease-, which cleaves the secretory form of IgA1, and regulating the expression of several genes encoding surface structures (LOS, pili, HMW and HMW2 proteins) through phase variation, that is the reversible loss or gain of a defined structure [39, 41, 42]. Beyond phase variation, selected surface molecules, such as P2 and P5 outer-membrane proteins, undergo antigenic drift, an irreversible process that involves substitution, deletion or addition of amino acids in their immunodominant regions [43].

13.2 Molecular Detection and Identification of *H. influenzae*

The name of the genus *Haemophilus* refers to the specific dependence of this organism on heme-related molecules for growth. *H. influenzae* is a fastidious organism and its bacterial growth requires a medium that includes NAD (V factor) and hemin (X factor) at 3–5% CO_2 environment and temperatures between 35 and 37°C. Culture methods for detection of *H. influenzae* include isolation of the microorganism from clinical specimens on appropriate supporting-growth medium (e.g., Chocolate agar), followed by identification based on requirement for both V and X factors and other biochemical characteristics [1]. In culture from the upper respiratory tract, the addition of bacitracin (300 mg/L) to the medium avoids the problem of overgrowth by concomitant bacteria.

Although the traditional culture methods for detection of *H. influenzae* from clinical specimens remains the "gold standard" reference method allowing the establishment of a collection of strains on which further phenotypic and genotypic investigations

can be performed, including the assessment of antibiotic resistance, it takes up to 48 h or more. Therefore, efforts have been spent to develop alternative rapid and accurate detection/identification methods, which are particularly valuable in diagnosis of life-threatening disease, such as meningitis, where survival is dependent on rapid diagnosis and early treatment. Moreover, diagnostic molecular methods that do not require bacterial growth are essential to increase diagnostic sensitivity in patients with culture-negative bacterial meningitis due to initiation of antibiotic therapy.

The first nonculture methods used in the diagnosis of Hib meningitis were based on direct detection of the soluble type b capsular polysaccharide antigen in Cerebrospinal fluid (CSF) of patients suspected of having bacterial meningitis using the counterimmunoelectrophoresis, enzyme-linked immunosorbent assay, coagglutination and latex agglutination (LA) techniques [44, 45]. All these techniques have a high sensitivity (minimum concentration of Hib polysaccharide antigen detectable has been reported to be 0.1–5 ng) and specificity and are able to provide true-positive results when nonviable organisms are present, but LA tests have several practical advantages over the others, since they are commercially available, rapid (≤15 min), and do not require special equipment. For these reasons LA tests have been widely employed in clinical microbiology laboratories. However, LA tests have the major setback that they can be used only for diagnosis of meningitis caused by *H. influenzae* type b strains, whereas encapsulated non-b and NTHi strains, to both which special attention is paid in the Hib post-vaccine era, cannot be detected. Obviously, LA tests remain a practical and reliable diagnostic tool in developing countries where Hib is still the leading cause of meningitis in children.

The availability of *H. influenzae* gene sequences and the advancements in DNA-based techniques, mainly PCR technology, have made it possible to apply the DNA-based methods for identification and/or direct detection of *H. influenzae* in clinical specimens. According to the European Union definition (Commission decision of 28/IV/2008 amending Decision 2002/253/EC), laboratory criteria for a case of *H. influenzae* meningitis/invasive disease are either (1) isolation of *H. influenzae* from a normally sterile site or (2) detection of *H. influenzae* nucleic acid from a normally sterile site. The latter may be achieved by direct amplification of target microbial DNA through PCR technique. It is well known that the sensitivity and mainly specificity of a PCR assay is determined by the target DNA sequence under evaluation. For detection of *H. influenzae* in CSF samples, the 16S ribosomal RNA gene, and the *bexA* gene are the most used targets.

H. influenzae can be identified by determining the sequence of the 16S rRNA gene and by comparing it with sequences of type strains available in databases accessible on the Internet (e.g., http://ncbi.nlm.nih.gov/BLAST), although some closely related species, such as *H. aegyptius* or *H. influenzae* biogroup aegyptius, cannot be differentiated from *H. influenzae* [1]. Sequencing of 16S rRNA gene is increasingly used to identify bacterial species in clinical practice, especially for detecting pathogens in normally sterile clinical specimens [46, 47]. To detect *H. influenzae* in CSF samples, the 16S rRNA gene PCR amplification followed by direct DNA sequencing of the amplicon is applied [48, 49]. Most of the steps, including DNA extraction, DNA amplification, purification of PCR products, DNA sequencing and sequence editing have to be performed manually, even if user-friendly

commercial kits for some of these steps are available and currently used for *H. influenzae* [50]. Since sequencing may be impractical in medical diagnostics where speed is essential, several efforts have been spent for setting up 16S rRNA-based methods in which the 16S rDNA PCR amplicon is subject to post-amplification analysis other than sequencing, through methods like further PCRs, restriction endonuclease digestions, and probing [51–54]. Semi-nested PCR strategy has been reported to be able to differentiate *H. influenzae*, *Streptococcus pneumoniae*, and *Neisseria meningitidis* in CSF specimens of patients with bacterial meningitis [51, 53]. Alternatively, a more recent and promising approach combines amplification of relatively short 16S rRNA gene sequences with specific detection of the DNA target through the use of hybridizing probes, in a real-time (RT) PCR [54–56]. Since no single 16S rRNA gene region, especially if short, as is the rule in RT PCR, can differentiate among all bacteria, the recent identification of the most appropriate 16S rRNA hypervariable region for *H. influenzae* undoubtedly provides a useful contribution in setting up these assays [57]. However, a limitation of the 16S rRNA gene-based methods is that they do not provide any information on the type (encapsulated or nonencapsulated) of *H. influenzae* strain detected, whereas this information is important to monitor invasive *H. influenzae* disease.

On the contrary, the target *bexA* gene, which encodes a protein involved in the polysaccharide export, is associated with capsulation and it has been successfully used for detection of Hib strains [22]. This target gene has been generally employed in multiplex PCR assays for simultaneous detection of *H. influenzae*, *Streptococcus pneumoniae*, and *Neisseria meningitidis* from CSF and blood, using both conventional and real-time PCRs [58–60]. Although the *bexA* gene is present in all encapsulated *H. influenzae* strains, recent studies revealed some polymorphism at nucleotide sequence level among different *H. influenzae* serotypes, that could affect the sensibility of the detection of some capsular types, including type f and e, resulting in a possible misidentification [61, 62]. A further limitation of using the *bexA* target is that NTHi strains are not detected since they do not actually possess this gene.

Finally, the gene encoding the outer-membrane protein P6, known to be conserved among encapsulated and nonencapsulated strains, has been proposed to be a target for detection of *H. influenzae* in CSF specimens, but no extensive studies on sensitivity and specificity of this detection method has been conducted [63, 64].

As reported above in the first paragraph of this chapter, *H. influenzae,* mainly NTHI, can cause acute respiratory tract infections. Indeed, there are limited data on the use of molecular techniques, such as PCR, for diagnosis of respiratory *H. influenzae* infections. Since *H. influenzae* is known to colonize the human respiratory tract and PCR technique has the potential to generate billions of copies of target DNA, establishing the clinical significance of a positive PCR result is a challenging task, apart from those cases in which detection of *H. influenzae* DNA occurs in normal sterile sites (pleural fluid or blood in diagnosis of pneumonia) [65–67]. However, a 16S rRNA gene-based approach has been employed to detect *H. influenzae*, together with other bacterial pathogens, in clinical samples of patients with community-acquired pneumonia or in sputum samples of cystic fibrosis patients [68–70]. The gene encoding P6 outer-membrane protein has been used as a target for *H. influenzae* detection in nasopharyngeal secretions and middle ear effusions [71, 72].

13.3 Typing Methods

Careful characterization of strains is a key factor in understanding and controlling disease caused by *H. influenzae*. First of all, *H. influenzae* strains are classified according to their capsular serotype, therefore this topic will be the subject of a separate paragraph.

13.3.1 Capsular Serotyping

Since the differences in pathogenic potential of nonencapsulated and individual serotypes of encapsulated strains of *H. influenzae,* capsule detection and serotyping have played a pivotal role in characterization of clinical isolates. As mentioned above, following widespread infant vaccination with conjugate Hib vaccines, the relative importance of encapsulated non-b and nonencapsulated (NTHi) strains has increased. To promptly detect potential "replacement disease," it is essential that laboratories supporting surveillance networks are able to distinguish between vaccine-preventable and non preventable strains. Moreover, an accurate serotyping system is particularly valuable for isolates from vaccine recipients, since a case of vaccine failure is defined as isolation of a *H. influenzae* type b strain in a previously vaccinated infant or child [73].

Traditionally, serotyping is carried out by slide agglutination method using polyvalent and type-specific antisera. The first step is determining whether a *H. influenzae* strain is capsulate or not by agglutination with polyvalent antiserum. If a strain is positive, further agglutinations with types a-f antisera are performed. A strong and rapid reaction with only one antiserum and without autoagglutination was required to record a test as positive. Antisera for *H. influenzae* serotyping are commercially available. Other non DNA-based serotyping methods include coagglutination with latex particles coated with type-specific antibodies and immunofluorescence microscopy [74]. Although slide agglutination has been for a long time commonly used by most clinical laboratories, it presents some limitations. Some strains exhibit either nonspecific agglutination or autoagglutination and strains expressing limited amount of capsule may not be identified [74–76]. Moreover, the discovery of spontaneously occurring capsule-deficient mutants of serotype b strains, named b- strains, in which the usually duplicated *cap b* locus undergoes a recombination event resulting in a single copy of *cap* genes and the loss of the *bexA* gene necessary for capsule expression, was a further issue calling for the development of capsular genotyping methods, since these b- strains are indistinguishable from NTHi strains by slide agglutination [77].

The first capsular genotyping of *H. influenzae* used a DNA probe-based technique, able to differentiate between the six serotypes in a Southern blotting procedure [78, 79]. However, it was with the application of the PCR to the detection of capsule-specific DNA that capsular genotyping become easy to approach. The PCR capsular typing method developed by Falla et al. includes different amplification steps [80]. First, primers specific for the *bexA* gene were used to differentiate between

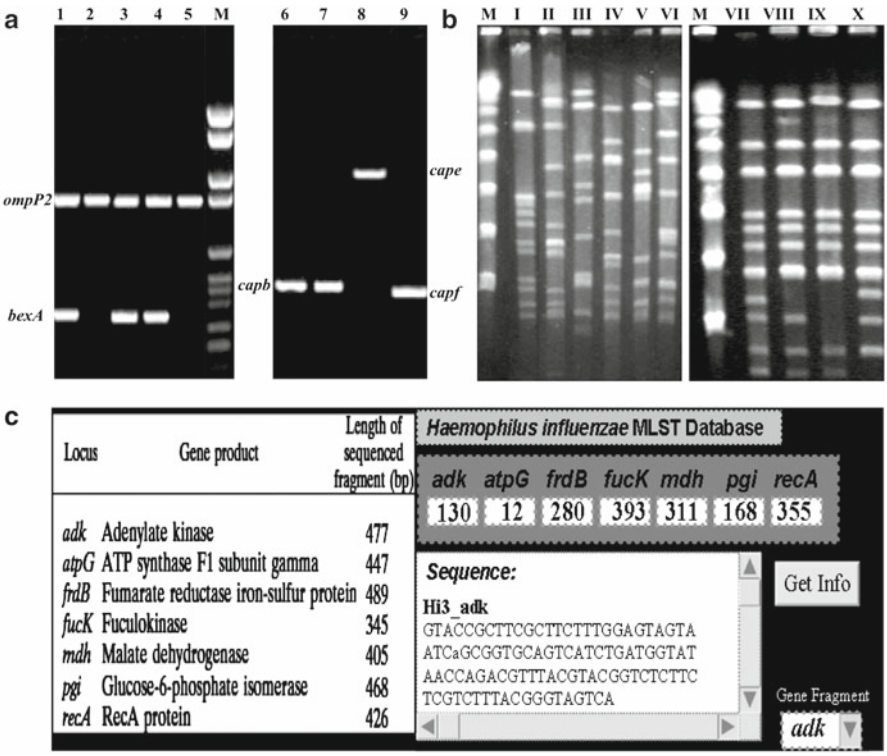

Fig. 13.1 The most used DNA-based methods for *Haemophilus influenzae* typing: PCR capsular typing, PFGE and MLST analysis. (**a**) Examples of PCR capsular typing; in the first round of PCR, the presence of a 1 kb product (*ompP2* gene) confirmed the species while the generation of a 343 bp product (*bexA* gene) denoted capsulation. In the second round of PCR, primers directed to each capsule type-specific region allowed us to identify the capsular type. A positive reaction with primers to *cap b* and a negative reaction with primers to *bexA* was a feature of b- strains. Lanes: 1, Hib; 2, b-; 3, type e; 4, type f; 5, NTHi; M, DNA Molecular Weight Marker X (Boeringer Mannheim); 6, Hib; 7 b-; 8, type e; 9 type f. (**b**) Examples of PFGE patterns obtained after digestion of chromosomal DNA with *SmaI* restriction enzyme. Lanes: M, Lambda Ladder PFG Marker (New England BioLabs); I through VI, NTHi strains; VII through X, Hib strains. (**c**) Schematic representation of the *H. influenzae* MLST Web site, showing the seven housekeeping genes, which together define the allelic profile or sequence type

encapsulated and nonencapsulated strains. Next, primers sets specific for each of the six capsular types were employed in separate PCRs to determine the capsular type. This method can be performed directly from colonies grown on suitable agar medium and b-strains can be unequivocally differentiated from NTHi. A later modification of the above protocol includes primers directed to the *ompP2* gene encoding the outer membrane protein P2, which is present in both encapsulated and nonencapsulated strains, to confirm *H. influenzae* species (Fig. 13.1a) [81, 82]. Several studies have demonstrated that PCR capsular typing is more sensitive and specific than conventional slide agglutination serotyping, since the most common error of the latter is the

misidentification of NTHi as encapsulated strains probably as result of nonspecific agglutination reactions [83, 84]. Recently, a two-step real time PCR assay has been evaluated for capsular typing of *H. influenzae* strains [85]. It included a first multiplex real time PCR test targeting a conserved region of the *bexA* gene, the region II of the *Hib* cap locus and the *ompP2* gene, and a subsequent real time PCR assays detecting capsule-specific DNAs for strains other than b. A high agreement (98%) was found comparing the real time PCR method with the gel-based PCR by Falla et al., making the first attractive due to its shorter turnaround time [85].

13.3.2 Other Typing Methods

Apart from capsular serotyping, a number of other methods have been employed to discriminate between *H. influenzae* strains by using several different markers. The earliest typing methods were based upon their phenotypic characteristics while, in recent years, genotypic techniques have received increased attention as a means a of investigating genetic relatedness among strains and monitoring the evolution of the bacterial population.

Among the phenotypic typing methods, biotyping has been traditionally used to subdivide *H. influenzae* strains into eight biotypes or biovars on the basis of indole production, urease and ornithine decarboxylase activities [1, 86]; however, the utility of biotyping in epidemiologic studies is limited, since it exhibits a low discriminatory power, especially among strains belonging to the same capsular type [86]. Other phenotypic methods for subtyping *H. influenzae* strains include outer membrane proteins analysis and lipopolysaccharide profiles [79, 87, 88], but it has been with the application of the multilocus enzyme electrophoresis (MLEE)—a method that classifies bacteria on the basis of the isoforms of a combination of about 15 essential metabolic enzymes—that, for the first time, evolutionary relationships between *H. influenzae* strains could be investigated [89]. Although MLEE is not strictly a DNA-based method, the polymorphisms in essential metabolic enzymes reflect changes in DNA sequences and are used to measure genetic divergence from a hypothetical common ancestor. Based on MLEE, encapsulated strains are clonal and can be segregated into genetically related clusters, which are grouped into two major phylogenetic divisions, I and II [90, 91]. Division I comprises the majority of clusters of both serotype a and b strains and all clusters of serotype c, d and e strains. Division II includes all clusters of serotype f strains and some minor clusters of serotype a and b strains. On the contrary, the population structure of NTHi has been found to be not clonal and NTHi strains seemed to exhibit considerable genetic heterogeneity [92, 93].

After the above reported pivotal studies, MLEE has been not widely used in characterization of *H. influenzae* strains, probably because it is low throughput and requires intensive laboratory work, with some noteworthy exceptions [94, 95]. On the other hand, the availability of the *H. influenzae* genome sequence as a target source for genotyping and the advances in molecular techniques have made the use of DNA-based

typing methods, such as ribotyping, enterobacterial repetitive intergenic consensus (ERIC) typing, multiple locus variable number tandem repeats analysis (MALVA), pulsed-field gel electrophoresis analysis (PFGE), and multilocus sequence typing (MLST), quite easy to apply to the study of *H. influenzae* infections.

Typing targeting of the rRNA genes is referred to as ribotyping. This method relies on variations within the 16S-23S-5S spacer regions of the ribosomal operon and it has been successfully used for typing a variety of bacterial species. For *H. influenzae*, ribotyping has been carried out with three different techniques: (1) restriction fragments length polymorphisms analysis of chromosomal DNA by Southern blotting, using rRNA or rDNA as a probe (conventional ribotyping), (2) amplification by PCR of 16S rDNA followed by restriction analysis of the PCR product (PCR-ribotyping), and (3) amplification by long PCR of a 6-kb region of the ribosomal operon followed by restriction analysis with *HaeIII* enzyme (Long PCR-ribotyping) [79, 96–101]. Ribotyping has been applied in epidemiological investigations of invasive *H. influenzae* infection as well as in studies on carriage, but it has been shown to be more useful in characterization of NTHi strains, in particular Long PCR-ribotyping has been specifically developed for this use [97]. By ribotyping, together with other typing methods, the high genetic heterogeneity of the NTHi strains has been demonstrated for the first time [99]. Compared with PFGE, ribotyping exhibits less discriminatory power and, currently is not extensively used [100]. Recently, sequencing of the 16S rRNA gene (16S typing) has been proposed for typing NTHi strains as a complementary approach to MLST [102].

ERIC sequences are conserved regions of DNA dispersed throughout the genome of Gram-negative, enteric bacteria. Since distribution of ERIC sequences varies between strains, PCR using ERIC-specific primers produces genetic fingerprinting of bacterial genome [103]. Actually, ERIC-PCR typing has been little applied to *H. influenzae* [99, 100, 104].

Variable number tandem repeats (VNTR) typing utilizes a feature of many bacterial genomes, including H. *influenzae*, which contain short repetitive tandem sequences strongly varying in overall number per locus and among unrelated strains. Analysis of VNTR polymorphism in *H. influenzae* strains is performed by PCR followed by determination of number of repeats for the different loci by visual inspection of the resolved PCR fragments [105, 106]. MALVA represents a technological advance in VNTR typing. After PCR with fluorescently labelled primers, fragments are separated on automated sequencer and analyzed by a software to perform sizing and to calculate number of repeats. MALVA was used to genotype Hib strains collected before and after introduction of Hib vaccination in Netherlands [107]. In contrast with results obtained by using other genotyping methods such as PFGE (see below), an increase in genetic diversity of Hib strains isolated from neonates and young children, but not in patients older than 4 years, after introduction of vaccination, was revealed by MALVA. It has been suggested that rather than to the emergence of a new Hib clone this increased diversity may be due to the fact that young children no longer constitute the reservoir for Hib and are infected by adults carrying genetically diverse Hib strains [107].

PFGE analysis compares the patterns of genomic DNA digested with a rare cutting restriction enzyme and it is considered a powerful discriminatory tool for distinguishing between *H. influenzae* strains. At present, this method is regarded as the "gold standard" for typing *H. influenzae* (Fig. 13.1b). Four restriction enzymes, *SmaI*, *ApaI*, *NaeI*, and *EagI*, were found to produce distributions of DNA fragments sizes useful for mapping *H. influenzae* genome by PFGE, but *SmaI* is generally used since it yields a smaller number of well-resolved fragments, with the exception of type e strains for which *ApaI* enzyme is often employed [14, 108]. As usual for PFGE, analysis of macro-restriction patterns is performed either by visual inspection of a small number of strains or by using software programs, which are able to normalize banding patterns over multiple gels and store the data in databases, when a large number of strains are compared. Most software programs, such as Diversity Data base Fingerprinting Software (Bio-Rad Laboratories, Hercules, CA, USA) contain algorithms that allow for similarity analysis and clustering of strains, resulting in a dendrogram. PFGE has been used in a great number of studies concerning ongoing transmission of Hib disease in different settings, monitoring of Hib isolates from vaccine recipients, characterization of invasive non-type b isolates in the post-vaccine era, studies on *H. influenzae* carriage and colonization. Although the clonal population structure of Hib has been confirmed by PFGE, this method has been successfully used to differentiate between Hib strains, due to its high discriminatory power [109–118]. Until now, by PFGE, no evidence of the emergence of new Hib clones exhibiting a marked genetic diversity has been found following the routine immunization of infants with Hib conjugate vaccine in different settings [113, 115, 116, 118]. However, some authors observed changes in capsule genes, which are amplification of *capb* gene sequences, in Hib strains circulating in the post-vaccine era, including isolates from children with Hib conjugate vaccine failure [27, 118]. To establish the copy number of the *capb* locus, a method that combines PFGE and Southern blotting detection may be used [26]. Since *Kpn*I and *Sma*I sites flank the *capb* locus, the number of copies is determined by Southern blot analysis on the basis of the size of the PFGE restriction fragments obtained following digestion of the chromosome with *Kpn*I and *Sma*I enzymes and detected by a capsule type b-specific probe [27]. Application of PFGE typing to investigations on non-vaccine preventable strains has achieved interesting results. In the post-vaccine era, encapsulated *H. influenzae* strains circulating in different countries generally belong to a few clones, whereas NTHi strains are characterized by an extensive genetic heterogeneity, even if isolated from invasive sites, confirming results previously obtained by MLEE and other typing methods [15, 16, 35, 82, 93, 94, 99, 115, 118, 119]. Bearing in mind that NTHi is implicated in many chronic infections and acute exacerbations of patients with both chronic obstructive pulmonary disease (COPD) and cystic fibrosis, an important application of PFGE genotyping is to study the persistence of this bacterium in patients. Establishing clonality or lack of clonality among different NTHi strains isolated from the same patient in different time periods can shed light on the dynamics of long-term NTHi colonization as well as on its role in the episodes of acute exacerbations [120–122].

Recently, a MLST scheme has been developed to characterize encapsulated and nonencapsulated *H. influenzae* isolates [93]. MLST is a sequence-based genotyping method that can be considered the natural evolution of MLEE [123]. It is based on the partial sequences of several housekeeping genes, which are present in all isolates of a particular species. Each isolate is defined by the alleles at each of the sequenced housekeeping loci, which together define the allelic profile or sequence type (ST). This method has the great advantage that results can be accumulated in database and shared between different laboratories in different parts of the world. Compared with PFGE, MLST has been used to assess deeper phylogenetic relationships in bacterial population, since it genotypes strains by sequencing housekeeping genes, which, by definition, require some time to diversify. For *H. influenzae*, MLST is based on sequencing of internal fragments of the seven housekeeping genes, *adk*, *atpG*, *frdB*, *fucK*, *mdh*, *pgi*, and *recA* [93]. The MLST database containing the allelic profiles and all information about *H. influenzae* strains together with analysis software can be found on the *H. influenzae* pages of the MLST Web site (http://haemophilus.mlst.net/) (Fig. 13.1c). The *H. influenzae* MLST scheme has been validated by comparison with the MLEE in a study demonstrating clustering of encapsulated strains within serotype-specific clusters (a single monophyletic group, each for types c, d, e, and f, but three and two high divergent lineages for type a and b, respectively), which were clearly distinct in genotypes from NTHi strains appearing as a separate population [93]. Actually, the NTHi population structure has been further analyzed in following studies, which have added information to the MLST database [35, 124–126]. It is now possible to identify distinct phylogenetic groups within the NTHi population with clustering of strains into separate groups according in part to the occurrence of specific genetic loci [125]. As far as encapsulated isolates are concerned, MLST may provide useful information on potential capsular switching among *H. influenzae* strains; if strains belonging to different serotypes do not have any common housekeeping gene alleles, no capsule switching occurs [127]. Finally, it should be mentioned that, in clinical settings, further differentiation within major clones identified by MLST may be required, especially for the encapsulated non-b strains, which belong to a few serotype-specific clusters. In these cases, other molecular typing procedures, such as PFGE, may be more discriminating than MLST.

In addition to detection of *H. influenzae* for diagnostic purpose and to classification of *H. influenzae* strains into types according to a typing scheme, DNA-based techniques may be used in several fields of *H. influenzae* research, for further genetic characterization of strains. Molecular methods are usefully applied for detecting genetic elements that encode for mechanisms of antimicrobial resistance or virulence determinants as well as shedding light on mechanisms involved in modulation of expression of several genes. To deal with this topic is not within the scope of this chapter; however, it should be emphasized that the study of distribution of specific genetic traits between different *H. influenzae* strains may be considered another approach to type bacteria. Finally, just a short mention of the several investigations concerning the *IS1016* insertion element, since this sequence plays a key role in the capsulation of *H. influenzae* strains. As explained above, *IS1016* is associated with *H. influenzae* capsule genes cluster in division I strains and provides the molecular

substrate for duplication and further amplifications of the *cap* gene sequences [21]. In addition to studies performed on *IS1016* in Hib strains, other *H. influenzae* types have been extensively analyzed. In serotype a strains, the presence of the *IS1016-bexA* partial deletion has been associated with enhanced virulence of such strains circulating in the post-vaccine era [10, 17] Moreover, although NTHi strains by definition lack a capsule, the *IS1016* element has been found in a small subgroup of NTHi strains with characteristics more closely resembling those of encapsulated *H. influenzae* [84, 128, 129].

13.4 Conclusions

The advent of the DNA-based techniques has opened a new era in all fields in the study of *H. influenzae*. It is worthy to remind that *H. influenzae* was the first free-living bacterium to have its genome completely sequenced, in 1955. Since then the availability of *H. influenzae* gene sequences has hugely increased, providing a invaluable tool in both molecular detection and molecular epidemiology of *H. influenzae*. In the Hib post-vaccine era, careful characterization of the non-b isolates circulating in different country is needed to promptly detect any possible replacement phenomena and any genetic modification, which may lead to emergence of hypervirulent strains. In the coming years, integration of molecular approaches in *H. influenzae* typing with the development of automation in the laboratory will strongly improve the monitoring and the control of *H. influenzae* disease.

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Chapter 14
Moraxella

Nevada M. Pingault and Thomas V. Riley

14.1 Introduction

The genus *Moraxella* consists of at least 15 species—including *Moraxella catarrhalis*, *Moraxella bovis*, *Moraxella lacunata*, *Moraxella osloensis*, *Moraxella nonliquefaciens*, *Moraxella atlantae*, *Moraxella lincolnii*, *Moraxella ovis*, *Moraxella caviae*, *Moraxella canis*, *Moraxella equi*, *Moraxella caniculi*, *Moraxella caprae*, *Moraxella boevrei* and *Moraxella bovoculi*. Members of this genus are generally Gram-negative bacilli (although *M. catarrhalis* demonstrates a diplococcoid arrangement), asaccharolytic, oxidase positive, mesophilic, non-fastidious, and they grow in air [1]. The predominant species of human importance is *M. catarrhalis*. This species, which was previously considered a commensal organism, is now a firmly established pathogen. While most commonly implicated in upper and lower respiratory tract infections, *M. catarrhalis* can also cause a host of other infections including bacteremia, endocarditis, conjunctivitis, meningitis, mastoiditis and septic arthritis [1]. It is the third most common bacterial cause of otitis media after *Streptococcus pneumoniae* and *Haemophilus influenzae*. Other *Moraxella* spp. are generally commensals in either humans or animals and are less commonly associated with

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disease. *M. lacunata* has been shown to cause conjunctivitis, keratitis, chronic sinusitis and endocarditis in humans; while *M. bovis* and *M. bovoculi*, and *M. ovis* have been implicated in bovine and ovine conjunctivitis, respectively.

Members of the *Moraxella* genus are biochemically homogenous, that is, they demonstrate a lack of biochemical variability. As such, the majority of phenotypic typing methods do not provide a sufficient level of discrimination between isolates for an epidemiological investigation. A host of genetic typing methods have been employed in recent years to further elucidate the molecular epidemiology of infections with this genus. The majority of investigations have been performed on *M. catarrhalis* which are the focus of this chapter.

Previous studies have shown *M. catarrhalis* to be a genetically diverse species. Care must be taken when choosing a genotyping method as the method must reflect what the researcher is attempting to demonstrate. If the researcher is trying to demonstrate broad epidemiological groupings, a less discriminatory typing method is advisable. However, if a researcher is looking for any degree of genetic variation then a more discriminatory method should be used. Typing methods for any organism must be assessed against a number of performance and convenience criteria including typeability, reproducibility, ease of performance and ease of interpretation [2]. Sufficient discriminatory power is also required for molecular methods. It is generally accepted [3] that a discriminatory index of 0.9 or above is required for reliable molecular typing. The following chapter reviews the methods that have been used for the molecular typing of *Moraxella* spp.

14.2 Typing Methods

14.2.1 MALDI-TOF

Matrix assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry (MS) is a method that has previously been used to analyse bacterial proteins and whole, intact bacterial cells resulting in the production of a mass spectral 'fingerprint' [4]. Previous research has distinguished two subpopulations of *M. catarrhalis* based predominantly on the complement resistance of the organism and 16S rRNA type [5]. Intact cell MALDI-TOF MS (ICM-MS) was used to differentiate between 16S rRNA subpopulations in a group of 18 *M. catarrhalis* isolates [4]. This technique was able to distinguish all Group 1 16S rRNA *M. catarrhalis* isolates from Group 2 and 3 isolates. In general, the technique was also able to distinguish between the Group 2 and 3 16S rRNA isolates. In 30% of Group 2 and 3 isolates the 16S rRNA type was incorrectly assigned. The method reportedly demonstrated excellent intra-strain reproducibility [4]. While this method is a protein based technique, it has been included in this chapter as it allowed distinction of rRNA types. This method would be classified as having low discriminatory power as it divides isolates into two broad genomic groups.

14.2.2 MLST

A multi locus sequence typing (MLST) scheme for *M. catarrhalis* was launched in 2003. The MLST database and methodology is accessible at http://mlst.ucc.ie/mlst/dbs/Mcatarrhalis. This MLST scheme uses eight housekeeping genes (*glyRS* [glycyl-tRNA synthetase beta subunit]; *ppa* [pyrophosphate phospho-hydrolase]; *efp* [elongation factor P]; *fumC* [fumarate hydratase]; *trpE* [anthranilate synthase component I]; *mutY* [adenine glycosylase]; *adk* [adenylate kinase]; and *abcZ* [ATP-binding protein]) that are distributed around the *M. catarrhalis* genome. Sequence data from 268 *M. catarrhalis* isolates collected worldwide were used to study ancestral relationships and virulence evolution. Using population genetic tools it was determined that the more virulent seroresistant lineage demonstrated higher mutation rates and homologous recombination rates in the housekeeping genes compared with the less virulent serosensitive lineage of *M. catarrhalis* [6]. MLST is classified as a library (definitive) method, meaning previous historical isolates can be compared with current isolates [2]. MLST is appropriate for examining long term epidemiological trends with *M. catarrhalis* as changes to housekeeping genes occur at a relatively slow rate. As more researchers submit information to the database it will allow more extensive analysis of *M. catarrhalis* population genetics. As of 28 August 2012 there are 335 isolates on the database, with 215 sequence types. A centrally maintained yet publicly accessible database is advantageous for the rapid worldwide dissemination of information. A downside to this method is that it generally requires access to a sequencer, the cost of which may be prohibitive to some laboratories.

14.2.3 Ribotyping

Ribotyping involves the digestion of bacterial DNA with restriction endonucleases followed by rRNA probing. This method can either be performed as a series of individual manual steps or using a fully automated system such as the Qualicon™ Riboprinter®. Manual ribotyping with a combination of *Hind*III and *Pst*I identified five ribotypes amongst 94 isolates of *M. catarrhalis* collected from 25 children with secretory and acute otitis media [7]. Two studies using automated ribotyping have been published. The first found 13 ribotypes amongst 28 complement sensitive *M. catarrhalis* strains and two ribotypes amongst 47 complement resistant strains when using *Eco*RI [8]. The second study identified four ribogroups in 25 *M. catarrhalis* isolates using *Pst*I, resulting in a discrimination index of 0.690 [9]. The degree of discrimination seen between *M. catarrhalis* isolates when using ribotyping is somewhat dependent on whether the isolates are complement sensitive or resistant. A higher level of discrimination is seen between complement sensitive isolates, while a lower level of discrimination is seen with complement resistant isolates. The rapid turnaround time of the automated method is advantageous, particularly in an outbreak situation and automated systems are extremely easy to use, not labour intensive and provide reproducible results. The RiboPrinter® system incorporates a

'dynamic' database, meaning that with each new pattern added to the system all previously submitted samples are also reanalysed. This allows patterns to be renamed if subtle differences are detected. Ribotypes generated by the RiboPrinter® system are able to be compared to patterns within the DuPont identification library. Unfortunately only a relatively small number of *M. catarrhalis* riboprint patterns are contained within the identification library. Automated ribotyping systems are prohibitively expensive for routine use for most laboratories, both in terms of initial cost of the apparatus and ongoing cost of maintenance and consumables.

14.2.4 RFLP, Probed RFLP and PCR-RFLP

In the simplest form, restriction fragment length polymorphism (RFLP) analysis involves the digestion of bacterial DNA with restriction enzymes followed by gel electrophoresis to produce DNA banding patterns. This method has been widely used in the past for the molecular typing of *M. catarrhalis* [10–12]. While a variety of restriction enzymes were trialled using this method (including *Hind*III, *Taq*I, *Pst*I, *Eco*RI, *Bam*HI and *Cla*I), researchers suggested that *Hae*III and *Hinf*I gave superior results to other restriction enzymes in relation to level of discrimination and readability [10–12]. RFLP is relatively quick, easy and inexpensive to perform, and generates reproducible results. There are two major disadvantages to this method. First, conventional electrophoresis is restricted to DNA fragments sized between ~0.2 and 20 kbp. Second, the restriction enzymes used are generally frequent cutters, resulting in the production of large numbers of bands. This in turn makes it difficult to interpret differences in banding patterns between isolates, especially if computer based software packages are not used.

PCR-RFLP entails PCR amplification of bacterial DNA using primers targeting specific genes or gene fragments, followed by digestion with restriction enzyme(s). The most commonly used primers for PCR-RFLP of *M. catarrhalis* are those for M46, a *M. catarrhalis* specific DNA fragment which encodes genes for glycyl tRNA synthetase [13]. These studies then used *Hae*III and *Rsa*I to digest the resulting amplicons. All studies demonstrated a moderate level of discrimination between isolates [8, 13, 14]. The use of specific primer(s) followed by restriction enzyme digestion results in easy to interpret banding patterns compared to conventional RFLP. PCR-RFLP has the advantage of not requiring high quality or concentration bacterial DNA, is relatively inexpensive and not overly labour intensive. M46 PCR-RFLP could be appropriate for short term epidemiological studies as the level of variation seen between isolates could determine clonality between strains [13]. Multi locus PCR-RFLP could be used for more long term epidemiological studies, as the degree of discrimination between isolates would be similar to using a multiprobe RFLP approach [13]. However, each added locus also increases the expense, labour intensiveness and complexity of interpreting the results of this method.

Probed RFLP involves either conventional RFLP or PCR-RFLP followed by Southern blot hybridisation with labelled probe(s). A study comparing probed RFLP

with conventional RFLP with *Hae*III determined that while probed RFLP was highly specific, it was less discriminatory than conventional RFLP, suggesting probed RFLP is best suited as a screening method [10]. Probed RFLP has also been used to successfully distinguish between complement resistant and sensitive *M. catarrhalis* strains [14]. Using this method is considerably more expensive and labour intensive than the simple serum based methods for detecting complement resistance [15]. A comparison between single locus PCR-RFLP and multiprobe RFLP [13] showed the latter was more discriminatory. These studies suggest probed RFLP is more labour-intensive, time-consuming and expensive than either conventional RFLP or PCR-RFLP, but results were easier to interpret. The level of discrimination provided by probed RFLP is dependent on whether a single or multi probe approach is used.

14.2.5 PFGE

Pulsed field gel electrophoresis (PFGE) is similar in principle to RFLP in that bacterial DNA is digested with restriction enzymes and electrophoresed resulting in the generation of banding patterns. However, PFGE uses infrequent cutting enzymes resulting in fewer bands and uses changing electric fields during electrophoresis meaning that larger DNA bands can be resolved. The first published use of PFGE for typing *M. catarrhalis* was in 1994 [16] and this study trialled numerous restriction enzymes that had previously been recommended for low guanosine–cytosine content organisms [17]. It was determined that *Not*I and *Sma*I were the most suitable; however, it was noted that some isolates were refractory to digestion with *Not*I, suggesting this was due to modification (methylation) of the *Not*I restriction site [16]. Further studies [8, 18, 19] confirmed that *Not*I, *Spe*I and *Sma*I are the most appropriate restriction enzymes for typing *M. catarrhalis*. *M. catarrhalis* DNA digestion with *Sma*I tends to result in a smaller number of bands than with *Not*I and *Spe*I, suggesting that *Sma*I is more suitable for revealing broad genomic groups. Since the initial report of using PFGE for typing *M. catarrhalis*, it has become the preferred molecular typing method for this organism, and the majority of articles detailing molecular typing of *M. catarrhalis*, particularly the more recent ones, have utilised PFGE. PFGE has a major advantage over most of the other typing methods detailed in this chapter in that results are generally highly reproducible. The results generated are not subject to operator and equipment influences to the same degree as some of the PCR based methods. There is also potential for data sharing between laboratories if enzyme choice, switch times and run times remain uniform, in which case PFGE can be considered a library method. While the use of infrequent cutting restriction enzymes and the switching electrophoresis field results in a lower number of larger sized bands, interpretation of results can still be difficult. Introduction of computer packages such as Bionumerics (Applied Maths, Belgium) and Molecular Analyst (Bio-Rad, USA) has improved the ease of interpretation not only for PFGE

but other molecular typing methods. PFGE has been viewed as a relatively labour intensive and lengthy method, and *M. catarrhalis* is known to produce nucleases which can result in background smearing on gels. However, improvements to the method [20] have simplified the PFGE process, reduced turnaround time and reduced background smearing. PFGE showed a high level of discrimination compared with both automated ribotyping and RAPD (discrimination index 0.983 versus 0.690 and 0.294, respectively) [9], and a level of discrimination comparable to PCR-RFLP [13]. PFGE is useful for both medium term epidemiological studies and for outbreak investigations. PFGE of *M. catarrhalis* reveals a wide range of genetic diversity within this species and as such is most suited to studies wanting to demonstrate a very high level of discrimination between isolates. The initial cost of purchasing a PFGE apparatus is high compared with PCR and/or conventional electrophoresis and this can be prohibitive to some laboratories. However, once purchased the ongoing expenses for this technique are minimal.

14.2.6 PCR Fingerprinting: RAPD and rep-PCR

Random amplified polymorphic DNA (RAPD) analysis is a typing technique that involves PCR amplification of DNA using random or arbitrary primers. rep-PCR is a technique similar to RAPD but uses primers representing known conserved repetitive DNA sequences instead of arbitrary primers [21]. A number of studies using these techniques with *Moraxella* spp. have shown varying levels of discrimination. A rep-PCR study examining 57 *M. bovis* isolates collected over a 3-year period from cattle with infectious bovine keratoconjunctivitis distinguished two clusters containing five RAPD types with a high degree of similarity between types [22]. A study trialling a combination of rep-PCR and RAPD primers found two which successfully distinguished between 75 complement resistant and sensitive *M. catarrhalis* strains [8]. A study of 13 *M. catarrhalis* isolates comparing RAPD with PFGE found one primer out of the six tested gave the same level of discrimination as PFGE with *Not*I [23]. However, a later study [9] could not replicate these results using a sample of 25 *M. catarrhalis* isolates, finding RAPD provided a much lower level of discrimination compared to PFGE and automated ribotyping. In addition, that study also demonstrated problems with the reproducibility of the RAPD results. The lack of inter-laboratory and intra-laboratory reproducibility is a known problem with these techniques and can be due to numerous factors including batch-to-batch primer variation, DNA template–primer ratio, DNA source/extraction method, *Taq* source and thermocycler variation [20]. A further study used a combination of six primers, including three previously described [23] to distinguish variation between two isolates collected at different time points from three children, two otitis prone and one not prone to otitis media [24]. One child (otitis prone) carried the same RAPD type over the two time points, while the other two children showed strain variation over the two time points [24]. While strain variation was demonstrated in this study, it was generally small scale variation in the order of two to three band

differences. In addition, a number of isolates failed to amplify with various primers. RAPD and rep-PCR are generally methods of low discriminatory power. These two factors make these methods best suited to small scale, short term epidemiological studies where broad epidemiological groupings are required. These methods are, however, rapid, meaning that they could be used in the investigation of a small scale outbreak. Both methods are also inexpensive to perform as minimal equipment and reagents are required, which is an advantage to less affluent laboratories.

14.2.7 sAFLP

Amplified fragment length polymorphism (AFLP™) is a PCR based method used to examine variation in small chromosomal fragments that has been used successfully for the molecular typing of a number of bacterial species. Only one study has been published on the use of AFLP™ for the typing of *M. catarrhalis* using a modified method known as single adapter AFLP (sAFLP) [5]. While AFLP™ usually involves the use of two adapters, sAFLP only uses one, resulting in circular DNA fragments. In theory, this increases the variety of restriction enzymes that can be used and improves reproducibility [5]. The study examined run to run variability by triplicate testing a number of isolates and found 96–98.6% similarity between the same isolate analysed on different days. Hence, strains with >96% similarity were assigned to the same sAFLP type. This study demonstrated a high level of discrimination between isolates, but also showed major clusters corresponding to 16S rRNA type, complement resistance and epithelial cell adherence [5].

14.3 Final Considerations

Members of the *Moraxella* genus do not show adequate phenotypic variation between isolates to use such methods for epidemiological typing. As such we must rely on genotypic typing methods. Genotypic research on the *Moraxella* genus has predominantly been performed with *M. catarrhalis*. *M. catarrhalis* has been reclassified from a commensal organism to a true pathogen in the last 30 years. It is an important cause of otitis media in children and can cause a variety of other infections in the immunocompromised host. The population structure of *M. catarrhalis* was largely unknown before the advent of molecular typing techniques. In the early 1990s, researchers began utilising techniques such as RFLP, RAPD, ribotyping and PFGE to further elucidate the epidemiology of infections caused by this organism. In recent years, PFGE has become the method of choice for typing *M. catarrhalis* due to the level of discrimination the method provides and its reproducibility. The recent development of a MLST typing scheme for *M. catarrhalis* will allow evolutionary trends of this organism to be revealed. The power of MLST for *M. catarrhalis* will no doubt increase as more researchers add information to the database.

Table 14.1 Advantages, disadvantages and potential uses of different molecular typing methods for *M. catarrhalis*

	Advantages	Disadvantages	Potential uses/other information
MALDI-TOF [4]	Uses whole intact bacterial cells, reproducible	Low discrimination, require experience to interpret profiles	Demonstration of broad genomic groups relating to complement resistance and 16S rRNA type. Protein based method
MLST [6]	Library/definitive method, standardised, data sharing, highly discriminatory	Expensive, relatively low number of isolates listed in database at present	Phylogenetic studies, evolutionary trends. Power will increase as more data added to database
Automated ribotyping [8, 9]	Rapid, easy to use and interpret results, reproducible, dynamic analysis	Expensive, only moderate level of discrimination	Outbreaks. Discrimination dependent of complement sensitivity
RFLP [10–12]	Rapid, easy to perform, reproducible, choice of enzymes, inexpensive	Large number of bands of small size, difficult to interpret results	Medium term epidemiological studies. Not widely used since advent of PFGE
PCR-RFLP [8, 13, 14]	Inexpensive, relatively easy to perform, smaller number of bands, easier to interpret results than RFLP, doesn't require high quality or concentration of DNA	Single locus of lower discrimination than multi locus. Increasing loci increases expense and complexity of results	Single locus—short term epidemiological study. Multi locus—medium term epidemiological study
Probed RFLP [10, 13, 14]	Highly specific, multiprobe approach more discriminatory than PCR-RFLP	Single probe less discriminatory than RFLP. Labour intensive, time consuming, expensive	Single probe—screening method. Multi probe—medium term epidemiological study
PFGE [8, 9, 13, 16, 18–20]	Reproducible, potential for data sharing, easier to interpret results than RFLP, low ongoing expenses, highly discriminatory	More time consuming compared to PCR methods, initial cost of apparatus high	Medium term epidemiological studies, outbreaks. Currently method of choice
Rep PCR, RAPD [8, 9, 22–24]	Inexpensive, rapid, easy to perform, minimal equipment or reagents	Low discrimination, reproducibility problems	Small scale, short term epidemiological studies and outbreaks

It has been demonstrated by a variety of researchers that *M. catarrhalis* is a genetically diverse species. The molecular typing method chosen must reflect the purpose of the study to be undertaken. It is important to note that one typing method is not going to be the universal choice for all researchers. Instead, researchers must have a clear research question in mind as this will guide the choice of genotyping method. If expense is an issue or researchers only want to demonstrate broad genomic groups in a small sample size, then methods such as RAPD or rep-PCR may be appropriate. If the laboratory is faced with an outbreak, then a method which is rapid and able to discriminate between historical and outbreak isolates is required. In such instances PFGE or automated ribotyping may be appropriate. If a researcher is trying to elucidate the evolution of their isolates in relation to *M. catarrhalis* isolates from around the world, then MLST would be most appropriate. The advantages, disadvantages and potential usage of the methods discussed in this chapter are summarised in Table 14.1.

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Chapter 15
Legionella pneumophila Typing

Christophe Ginevra

Legionellae are Gram-negative bacteria that can cause sporadic cases and outbreaks of pneumonia when water droplets are inhaled from a variety of natural and man-made sources [1]. There are more than 50 different species of *Legionella* and although 20 are documented as human pathogens [1], up to 90 % of clinical cases are caused by *Legionella pneumophila*. Among the 15 serogroups (Sg) characterized within the species *L. pneumophila*, Sg1 is responsible for about 85 % of all cases worldwide [2, 3].

Typing of *L. pneumophila* has two principal applications.

- Identification of environmental sources of infections in order to prevent or to stop an outbreak. Comparison of clinical and environmental isolates is necessary to identify a water reservoir as the source of infections.
- Studying the dynamics of *Legionella* populations.

The first step of *Legionella* identification is its auxotroph character for cysteine. Legionellae have proved to be relatively unreactive when traditional biochemical tests are utilized, necessitating more complex identification methods. Specific antibodies are commonly used for rapid discrimination of *L. pneumophila* serogroup 1 from other *L. pneumophila* and from other *Legionella* in latex agglutination assays. They are also used for acute species identification (e.g., indirect immunofluorescence assay), but cross reactions are frequent and molecular techniques such as *mip* sequencing appear to be the gold standard for the species identification of *Legionella* non *pneumophila* [4]. Recently matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been described as a useful tool for *Legionella* species identification [5, 6].

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Usually typing techniques are applied on *L. pneumophila* serogroup 1 isolates; nevertheless, some of the typing methods described below can be applied on other *Legionella* isolates.

After genus and species identification, a first phenotypic screen can be performed by subgrouping *L. pneumophila* serogroup 1 using panels of monoclonal antibodies as described by Joly et al. and Helbig et al. [7, 8]. The latter described a standard phenotyping scheme allowing dividing Lp1 into 9 subgroups which is very low for a typing method but which constitutes a interesting first screen which could enhance the discriminatory power of an associated genotyping method [9].

The key point of molecular typing is the selection of the molecular marker. The marker has to be variable enough to differentiate two unrelated isolates, but stable enough to remain identical between a same strain isolated on one side from patients' samples during an outbreak and on the other side from environmental samples during epidemiological investigations.

For *L. pneumophila*, most molecular typing methods are based on comparison of DNA banding patterns generated by several methods. More recently, sequence-based methods have been developed [9–13]. A MALDI-TOF-MS approach has also been described recently [14].

To facilitate the monitoring of travel associated LD, a European group was created in 1986 European working group on *Legionella* infection (EWGLI). This group has worked on the standardization at an international level of molecular typing methods.

Restriction fragment length polymorphisms (RFLP) is one of the first methods developed for *L. pneumophila* molecular typing and has been used as the gold standard until recently in some countries such as the UK in which it was used for at least 19 years [15]. The method is based on probing restriction fragments of chromosomal DNA with cloned probes composed of randomly selected regions of the *L. pneumophila* chromosome [16]. This method has also been use for *Legionella longbeachae* typing [17].

When the probe used for hybridization is derived from rRNA, the method is called ribotyping. Ribotyping was first used for *Legionella* species identification by using 16S–23S ITS probe [18]. It was then used for *L. pneumophila* subtyping using different probes [19].

RFLP typing has a high discriminatory index, but several methods developed later appear to have an equal discriminatory index but are easier to set up (e.g., PCR-based techniques) or have shown to be more discriminative (e.g., Pulse-field gel electrophoresis (PFGE) [20]).

PFGE is one of the gold standards for local epidemiology (e.g., outbreak investigation); this method has been used for several years. The method is based on the separation by pulse-field electrophoresis of macrorestriction fragments of the bacterial chromosome generated by digestion with an infrequent cutting site restriction endonuclease. PFGE has a high discriminatory index as assessed by several studies; most of these studies described the use of *Sfi*I restriction endonuclease [21–23]. Despite its high discriminatory index, this method suffers some drawbacks: it is time consuming (4 days to obtain results), inter-gel reproducibility is poor, electrophoresis requires specific equipment and computer-aided imaging analysis is

needed, data are difficult to exchange between laboratories making investigations of travel-associated LD cases harder.

Recently Chang et al. described an improved protocol for *L. pneumophila* typing reducing to 2 days the total duration of the experiment [24]. Based on the global genomes sequenced, Zhou et al. evaluated new restriction endonuclease for PFGE typing, they also optimized electrophoretic parameters [25]. Despite some international standardization of PFGE typing protocols (restriction endonuclease, plugs preparation, and electrophoretic parameters) data remain difficult to exchange.

PFGE has also been used for *L. longbeachae* typing [26]; moreover, an optimized protocol using a double digestion has been described for *Legionella anisa* typing [27].

Several authors have used arbitrary primed PCR (AP-PCR) for *L. pneumophila* subtyping [28–30]. This method is based on the generation of DNA finger printing by random amplification of genome fragments. These techniques allow a good discriminatory index, are easy to perform, and give rapid results. The major drawback for these techniques is the lack of reproducibility between laboratories. Several primers have been tested for *L. pneumophila* subtyping [29, 30] and some authors recommended combination of the results obtained with two different primers before drawing any conclusion about the relatedness between strains [29].

AFLP, one of the methods standardized by the EWGLI, has a high discriminatory index. In this method, bacterial DNA is simply digested and specific adapters are ligated to the restriction fragments. These adapters are then use as targets for PCR amplification. The length polymorphism of amplified fragments generated is visualized by agarose or acrylamide gel electrophoresis [31–33].

The infrequent-restriction-site PCR (IRS-PCR) assay was developed for *L. pneumophila* molecular typing by Riffard et al. and shows a high discriminatory index in his study, similar to that of PFGE [23]. IRS-PCR consists of double digestion of genomic DNA with a restriction enzyme that infrequently cuts the chromosome and a second enzyme that frequently cuts it, followed by amplification of DNA with primers and adapters targeting the extremities of the restricted fragments. This method has the advantage of using low quantities of target DNA, and the separation of amplified fragments can be achieved by conventional agarose gel electrophoresis.

Pourcel et al. first described the use of variable-number tandem-repeat (VNTR) diversity for *L. pneumophila* typing [34]. The authors developed this method based on the *L. pneumophila* Philadelphia genome and then updated it after the sequencing of two others strains (Paris and Lens) [11]. The method is based on the length polymorphisms of 8 VNTR regions. These VNTR are repetitive sequences and the number of repetitions vary between strains. The length polymorphisms can be easily visualized by PCR amplification of each locus using flanking primers and electrophoresic migration. This technique is called MLVA for multi-locus VNTR analysis. The MLVA type or profile is composed of a string of allele numbers, corresponding to the number of repeats at each VNTR locus, separated by commas, in a predetermined order. *L. pneumophila* MLVA typing has been adapted to an automated multicolored capillary electrophoresis in a multiplex assay by Nederbragt which made the method more accurate and more sensitive than the gel-based method

[35]. An MLVA-type database has also been created and is available on the website http://bacterial-genotyping.igmors.u-psud.fr/.

Conversely to AP-PCR, the amplification step of AFLP, IRS-PCR, and MLVA is performed in stringent conditions which made inter-laboratories reproducibility higher.

Several methods are based on the comparison of polymorphisms of several DNA fragments.

The method is called multilocus sequence typing (MLST) when targets sequences are parts of housekeeping genes, sequence-based typing (SBT) which targets sequences are part of more variable genes, and multispacer typing (MST) when target sequences are highly variable intergenic region.

Since 2007, SBT is the new gold standard method recommended by EWGLI. This method is based on the sequence comparison of seven genes (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*). An allelic profile comprises a string of numbers encompassing the number of individual alleles of the genes separated by commas. Each allelic profile corresponds to a sequence type (ST) (e.g., allelic profile 1,4,3,1,1,1,1 corresponds to ST1) [9, 10]. The assignment of allelic profile and ST could be done by submitting the raw data sequences on the web interface available on the EWGLI website.

Isolation of clinical or environmental *L. pneumophila* is not easy. This impairs the epidemiological investigations as both clinical and environmental isolates are required for comparison to find the source of infection. SBT is a PCR-based method which can be applied directly to DNA extracted from clinical or environmental samples without isolates. This direct use of the method on samples as been published in few cases and gives variable results in some case all genes could be amplified and sequenced [36], in other cases no or few genes could be amplified and sequenced in environmental or in clinical samples [37]. To enhance the sensitivity of SBT directly applied on clinical samples, two studies have described the addition of a previous amplification step leading to nested or semi-nested PCR before sequencing of the target genes [38, 39]. In their study, Coscollá et al. enhanced the discriminatory index of the method by adding to the six first gene targets of the standard SBT 3 intergenic regions as new targets, but these targets were not included in the EWGLI definition of sequence types.

Fujinami et al. evaluated MALDI-TOF-MS for rapid discrimination of *Legionella* isolates [14]. The authors evaluated the use of MALDI-TOF-MS for *Legionella* species identification on one hand and *L. pneumophila* typing on the other hand. In their study, different *Legionella* species could be differentiated and two set of *L. pneumophila* isolates clustered in the same way when typed by mass spectrometry clusters or by PFGE. Nevertheless, in their study, the authors only tested 23 *L. pneumophila* isolates and nine other *Legionella* species. This method should be evaluated on a larger number of isolates from more diverse origins. If the high discriminatory index of MALDI-TOF-MS for *L. pneumophila* typing is demonstrated on a large isolate collection, this method will be a useful tool. MALDI-TOF-MS data can be generated within a few hours after *Legionella* growth. MALDI-TOF mass spectrometers are increasingly present in microbiology laboratories due to its increasing use for bacterial identification.

15.1 Conclusion

Several studies have compared these typing methods for discrimination indexes and also for rapidity, intra- and inter-laboratories reproducibility, and ease to exchange results.

PCR-based methods have the advantage of rapidity and their ease to be performed in standard laboratories and can be recommended for initial investigations during outbreaks.

PFGE remains a highly discriminative method, but it can be performed only by specialized laboratories such as reference centers. Results remain still difficult to exchange between laboratories. This method is recommended for local epidemiology investigation by specialized laboratories.

SBT appears to be the method of choice for the exchange of results and is a powerful tool for global epidemiology. The additions of new targets have demonstrated that the discriminative power of this technique can be enhanced easily [9, 36]. The possible application of SBT method directly on clinical and environmental samples offers new solutions during epidemiological investigations.

Enhanced molecular characterization of Legionellae by SBT coupled with techniques like monoclonal antibody testing or PFGE typing allows us to build up a global picture of strain distribution and significance. The collaborative results obtained by members of EWGLI since 2003 using an SBT scheme show that a minority of strains cause most disease. Several independent studies show that few genotypes (ST1, ST23, ST37, ST40, ST47, ST62, etc.) cause lots of culture-proven LD cases and that these genotypes could be worldwide spread.

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Chapter 16
Mycoplasma and Ureaplasma

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16.1 Introduction

The bacteria commonly referred to as mycoplasmas are included within the Phylum Tenericutes, Class *Mollicutes* which comprises 4 orders, 5 families, 8 genera, and about 200 known species distributed among humans, vertebrate animals, insects, and plants (Table 16.1). New species, mainly in animals, are still being identified and more are expected to be discovered in the future since many hosts have never been evaluated for the presence of parasitic mycoplasmas. There are 16 mollicute species isolated from humans on multiple occasions. This excludes species of animal origin that have been detected occasionally in humans from time to time, usually in immunosuppressed hosts, but which are generally considered transient colonizers. Among mollicutes of humans, there are at least six species believed to be of pathologic significance in some contexts, either as primary pathogens or opportunists: *Mycoplasma pneumoniae, M. hominis, M. genitalium, M. fermentans, Ureaplasma urealyticum,* and *Ureaplasma parvum.* A newly described species, *M. amphoriforme*, has been detected in the lower respiratory tracts of several

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Table 16.1 Characteristics of the class *Mollicutes*[a]

Order	Family	Genus	No. species	Sterols required	Hosts	Genome size (kbp)	Mol% G+C of DNA	Defining features
Mycoplasmatales	*Mycoplasmataceae*	*Mycoplasma*	>100	Yes	Humans & animals	580–1,350	23–41	
		Ureaplasma	7	Yes	Humans & animals	760–1,140	27–30	Metabolizes urea
Entomoplasmatales	*Entomoplasmataceae*	*Entomoplasma*	6	Yes	Insects & plants	870–900	27–29	
		Mesoplasma	11	No	Insects & plants	825–930	27–30	
	Spiroplasmataceae	*Spiroplasma*	37	Yes	Insects & plants	780–2,220	25–31	Helical morphology
Acholeplasmatales	*Acholeplasmataaceae*	*Acholeplasma*	18	No	Animals, insects & plants	530–1,350	27–36	
Anaeroplasmatales	*Anaeroplasmataceae*	*Anaeroplasma*	4	Yes	Animals	1,500–1,600	29–33	Obligate anaerobes
		Asteroleplasma	1	No	animals	1,500	40	Obligate anaerobes

[a]Mollicutes enumerated in this table do not include the noncultivable hemotrophic mycoplasmas and *Candidatus* Phytoplasmas

immunodeficient persons with respiratory disease, but there are no conclusive data thus far that this mycoplasma is a significant pathogen of humans [1]. This chapter will focus primarily on methods for detection, identification, and typing of pathogenic mollicutes of humans and will not include information on the many mycoplasmal pathogens of animals.

16.2 Class *Mollicutes*

Mollicutes are smaller than conventional bacteria, in cellular dimensions as well as genome size, making them the smallest free-living organisms known. It is believed that mollicutes constitute a phylogenetic lineage within the Gram-positive bacteria, evolving from clostridial-like ancestors through successive gene deletion. Their cell volumes are approximately 5% of that of a typical bacterium. Some species such as *M. pneumoniae* and *M. genitalium* also possess distinct terminal attachment organelles [2]. Mollicutes cannot be detected by light microscopy and they rarely produce visible turbidity in liquid growth media. They do not possess a cell wall, but like mammalian cells, they are bounded by phospholipid bilayer membranes. The permanent lack of a cell wall barrier makes mollicutes unique among prokaryotes and differentiates them from bacterial L forms for which the lack of the cell wall is but a temporary reflection of environmental conditions. Lack of a cell wall also renders mollicutes insensitive to the activity of beta-lactam antimicrobials, prevents them from staining by Gram's stain, and is largely responsible for their pleomorphic form. Most mollicutes are nonmotile, but some species show gliding motility. Mollicutes contain a single circular chromosome and a low G+C content (23–40 mol%). The extremely small genome and limited biosynthetic capabilities explain the parasitic or saprophytic existence of these organisms, their sensitivity to environmental conditions, and their fastidious growth requirements. Among the mollicutes that are important human pathogens, there have been at least one or more type strains for which the genome has now been completely sequenced and annotated. Genome sequencing and pulsed field gel electrophoresis data accumulated to date indicate mollicute genome sizes range from 580 to 2,200 kbp. Additional type strains and clinical isolates of important mollicute pathogens of humans are also undergoing complete genomic sequencing in order to provide a better understanding of the comparative genomics of these organisms and offer clues about their pathogenic mechanisms at the molecular level.

Mollicutes require enriched growth medium supplemented with nucleic acid precursors, fatty acids, and amino acids. Except for acholeplasmas, asteroleplasmas, and mesoplasmas, mollicutes require sterols in growth media, supplied by the addition of horse or bovine serum. Although these organisms can flourish in the osmotically stable environment of a eukaryotic host, they are extremely susceptible to desiccation. In addition to the phospholipid bilayer membrane, mollicute cells typically have an underlying protein cytoskeleton network that provides structural support. Some mollicutes, including *M. pneumoniae* and *Ureaplasma* spp. also elaborate extracellular

capsular material [3, 4]. Growth rates in culture medium vary among individual species, with generation times of approximately 1 h for *Ureaplasma* spp., 6 h for *M. pneumoniae*, and 16 h for *M. genitalium* [5]. Typical mollicute colonies vary from 15 to 300 μm in diameter. Colonies of some species, such as *M. hominis*, often exhibit a "fried egg" appearance owing to the contrast in deeper growth in the center of the colony with more shallow growth at the periphery, while others, such as *M. pneumoniae* and *M. genitalium* produce spherical colonies. Whereas colonies of some mycoplasmal species may be observed with the naked eye, those produced by ureaplasmas are typically 15–60 μm in diameter, and require low-power microscopic magnification for visualization.

Mollicutes of human origin can be classified according to whether they ferment glucose, utilize arginine, or hydrolyze urea. Except for hydrolysis of urea, which is unique for ureaplasmas, these biochemical features are not sufficient for species distinction. Anaeroplasmas and asteroleplasmas, which occur in ruminants, are strictly anaerobic and oxygen-sensitive, while most other mollicutes are facultative anaerobes. The type of media components, pH, metabolic substrates, and optimum temperature for *in vitro* cultivation and colony development can vary considerably for the many different species that may be encountered in various hosts. No tricarboxylic acid cycle enzymes, quinones, or cytochromes have been found in this class.

The 16S rRNA gene sequences place the mollicutes into several distinct phylogenetic groups (Fig. 16.1) Analysis of rRNA gene sequences has also become the cornerstone of the modern species concept of the mollicutes, which previously utilized DNA–DNA hybridization and serology, and is now essential for evaluation of novel species. The 16S rRNA gene sequence analyses have also shown that certain noncultivable hemotropic bacteria previously classified among the *Rickettsiae* belong to the Class *Mollicutes* [1].

16.2.1 Genus Mycoplasma

The genus *Mycoplasma* is by far the largest and most important in the class, comprising well over 100 known species, which may occur as commensals or cause significant diseases in vertebrate animals or humans. General characteristic of the genus *Mycoplasma* in comparison to other genera in the Class *Mollicutes* are listed in Table 16.1. Some important characteristics of individual *Mycoplasma* species that occur in humans are shown in Table 16.2. Emphasis in the following discussion will be placed on the four *Mycoplasma* spp. known to be pathogenic for humans: *M. pneumoniae, M. hominis, M. genitalium*, and *M. fermentans.*

16.2.1.1 *Mycoplasma fermentans*

M. fermentans can occur in many body fluids and tissues, including the urogenital tract, throat, lower respiratory tract, rectum, and joints in various patient populations [6], but its primary site of colonization, mode of transmission, and pathogenic

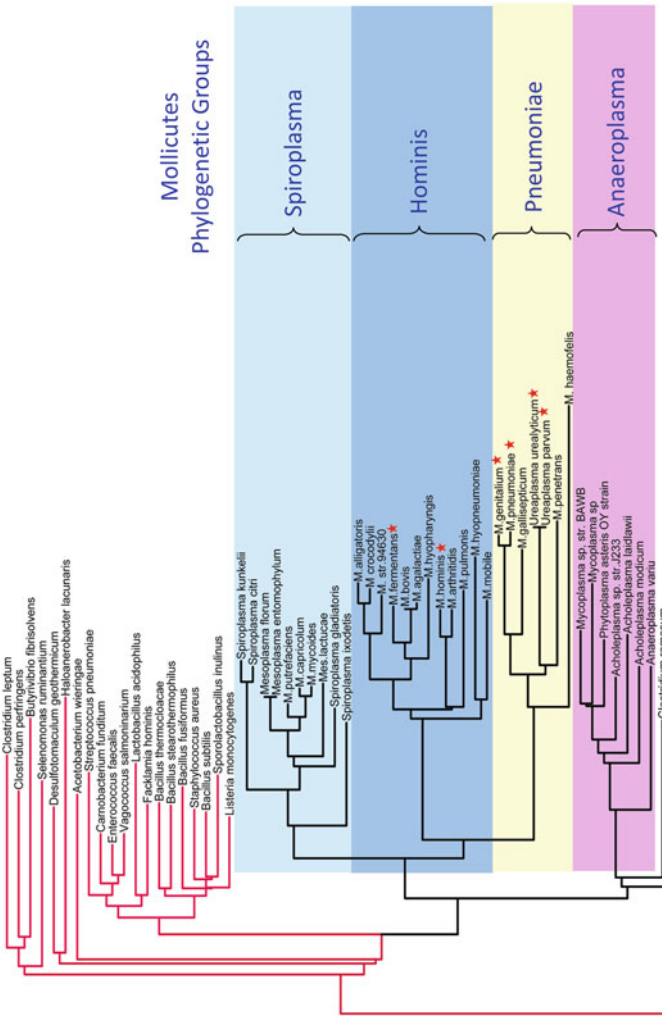

Fig. 16.1 Phylogenetic Classification of Mollicutes Based on 16S rRNA Gene. A dendogram showing Mollicute phylogeny based on 16S rRNA sequences is shown. The six species of *Mycoplasma* and *Ureaplasma* that are proven human pathogens and which are the subject of this chapter are noted with red stars. The four main taxonomic groups of the Class *Mollicutes* are highlighted in different background colors

Table 16.2 Characteristics of *Mycoplasma* species that colonize and infect humans

Species	Primary Site of colonization		Metabolic substrate		Role in human diseases[a]
	Respiratory tract	Urogenital tract	Glucose	Arginine	
M. amphoriforme[b]	+	–	+	–	?
M. buccale	+	–	–	+	No
M. faucium	+	–	–	+	No
M. fermentans	+	+	+	+	Yes
M. genitalium	?+	+	+	–	Yes
M. hominis	+	+	–	+	Yes
M. lipophilum	+	–	–	+	No
M. penetrans[c]	–	+	+	+	?
M. pirum[d]	?	?	+	+	No
M. pneumoniae	+	–	+	–	Yes
M. primatum	–	+	–	+	No
M. salivarium	+	–	–	+	No
M. spermatophilum	–	+	–	+	No

[a]Some of the species listed as commensals have very rarely been shown to produce an invasive disease in the setting of an immunocompromised host, but they are still considered nonpathogenic commensals overall

[b]*M. amphoriforme* is the newest human *Mycoplasma* species to be identified. It was recovered from the respiratory tract of several British patients with antibody deficiency and chronic bronchitis or bronchiectasis [183], and has subsequently been detected in other European countries and Tunisia. However, its occurrence in healthy persons and an independent role in human disease have not been rigorously established thus far

[c]*M. penetrans* has been detected in the urine, rectum and throats of homosexual men with human immunodeficiency virus infection. However, this mycoplasma has not been conclusively shown to produce significant disease in any patient population, despite the fact that it possess potentially pathogenic features such as a prominent terminal attachment organelle and has proven ability to invade host cells [6]

[d]*M. pirum* was first characterized in 1985, but its natural host was unknown. This mycoplasma has been isolated from peripheral blood lymphocytes and urines in persons with human immunodeficiency virus infection and has been found by PCR in the rectums of homosexual men. It has also been detected in healthy persons and no conclusive evidence that it is a cause of human disease has been forthcoming [6]

potential are incompletely understood. It has been detected in adults with an acute influenza-like illness [7], and in bronchoalveolar lavages, peripheral blood lymphocytes, and bone marrow from patients with the acquired immunodeficiency syndrome (AIDS) and respiratory disease [8, 9]. It is apparent that respiratory infection with *M. fermentans* is not necessarily linked with immunodeficiency, but it may also behave as an opportunistic pathogen. Several studies from multiple countries using suitable case and control populations employing culture, serology and/or PCR have implicated *M. fermentans* in a variety of human inflammatory arthritides including rheumatoid arthritis [10]. However, the possibility that the organisms may indeed be present, but unrelated to the arthritic condition has not been totally discounted. Considerable interest arose in the 1990s regarding the possibility that *M. fermentans* could be a cofactor in the pathogenesis in AIDS and/or as an etiologic agent of

fibromyalgia, Gulf War and Chronic Fatigue Syndromes. However, after several large scale studies failed to demonstrate a conclusive role for this organism in these conditions, the belief that *M. fermentans* is pathogenic in such situations has largely been abandoned [10].

M. fermentans is able to invade host cells and produces a potent immunomodulator, macrophage activating lipopeptide 2, which stimulates macrophages to release cytokines that may increase neutrophilic infiltration locally where the organism resides [10]. This mycoplasma does not have the specialized attachment organelle that occurs in some other species such as *M. pneumoniae.* Additional virulence factors may include plasminogen activation and other membrane surface proteins that mediate cell fusion, cytadherence and antigenic variation as discussed by Rottem [11].

The *M. fermentans* strain JER genome sequence showed the organism has a 978 kbp genome that encodes 798 proteins [12] *M. fermentans* grows well on SP4 or Hayflick's agar (pH 7.4–7.6) supplemented with either arginine or glucose since it has the enzymatic systems to utilize both as substrates. It produces fried egg colonies after several days of incubation. However, cultivation and growth conditions for this mycoplasma are not nearly as well defined as those for other common human pathogens such as *M. hominis* and *M. pneumoniae.*

16.2.1.2 *Mycoplasma genitalium*

M. genitalium was initially isolated from men with urethritis in 1980 and is now known to be a significant cause of this condition as well as female cervicitis, and pelvic inflammatory disease [13]. *M. genitalium* possesses a terminal structure, the MgPa adhesin, which facilitates its attachment to epithelial cells [14]. This mycoplasma also attaches to spermatozoa and erythrocytes, and invades epithelial cells with evidence of nuclear localization [13]. A family of repetitive DNA elements with homology to the MgPa adhesin gene provides a reservoir of sequence that could contribute to variation in the protein of the MgPa adhesin gene. Sequence divergence among strains of *M. genitalium* has been shown and this antigenic variation may help avoid the host immune response and optimize adhesion [15].

The 580 kbp genome of *M. genitalium* contains 485 protein coding genes, has a G+C content of 32% and the organism is glycolytic. Cultivation *in vitro* is difficult and time-consuming, requiring up to several weeks, although subcultures may grow more rapidly. The best culture medium is SP4 broth and agar incubated at 37°C in nitrogen or air plus 5% CO_2. Additional techniques designed to improve recovery of *M. genitalium* from clinical specimens have involved serial passages in Vero cells to allow adaptation for the organisms to grow in broth and eventually on agar [5]. Though various modalities have been developed to enhance the ability to detect *M. genitalium* in culture, the high failure rate and extremely slow growth make the culture approach impractical now that molecular-based assays have been developed.

16.2.1.3 *Mycoplasma hominis*

The organism that eventually came to be known as *M. hominis* was the first *Mycoplasma* species isolated from humans and associated with disease [16]. Approximately 21–53% of asymptomatic sexually active women may be colonized with this mycoplasma in the cervix or vagina, but the occurrence is somewhat lower in the male urethra [6]. It is often present concurrently with *Ureaplasma* spp. and is transmissible venereally and vertically. *M. hominis* is associated with a variety of conditions including pyelonephritis, pelvic inflammatory diseases, chorioamnionitis, post-partum endometritis, bacterial vaginosis, arthritis, osteoarthritis, wound infections, and several conditions in neonates including congenital pneumonia, meningitis, bacteremia, and abscesses. Systemic infections are usually, but not always, associated with immunocompromised hosts [6].

The *M. hominis* genome has been shown to contain 665 kbp with an overall G+C content of 27.1% and 527 protein coding genes [17]. Analysis of the genome indicated that this mycoplasma has undergone horizontal gene transfer with *Ureaplasma* spp. [17]. This genome size places it second, behind *M. genitalium* as the smallest known self-replicating free-living organism [17]. Henrich [18] demonstrated the presence of the variable adherence-associated antigen (Vaa), which displays high frequency phase and size variation that is believed to be a major adhesin of *M. hominis* and may also assist in evasion of host immune responses. Additional surface proteins such as OppA, an oligopeptide permease substrate-binding protein are also believed to be involved in cytadherence and may also induce ATP-release from cells, resulting in apoptosis [17].

M. hominis is a non-glycolytic mycoplasma. It synthesizes ATP through the arginine dihydrolase pathway [17]. It grows well in SP4 broth or agar medium (pH 7.4–7.6) supplemented with arginine at 37°C in 5% CO_2 in air, but it will also grow on A8 agar at pH 6.0–6.5 and in 10B broth. Colonies develop in 2–3 days and exhibit the typical fried egg appearance. Unlike other mycoplasmas that are pathogenic for humans, *M. hominis* will often grow on other bacteriological media such as Columbia agar and growth is enhanced by anaerobic incubation.

16.2.1.4 *Mycoplasma pneumoniae*

M. pneumoniae is a common cause of upper and lower respiratory tract infections in children and adults worldwide. It is easily spread through respiratory droplets and can cause a wide array of clinical manifestations including pharyngitis, tracheobronchitis, and pneumonia. Extrapulmonary manifestations involving any of the major organ systems sometimes occur following primary respiratory infection either by direct spread or autoimmune effects [2, 19]. Attachment of *M. pneumoniae* to host cells in the respiratory tract of humans is a prerequisite for colonization and infection. Cytadherence is mediated by the P1 adhesin and other accessory proteins, described in detail elsewhere [2, 19] and is followed by induction of chronic

inflammation, and cytotoxicity mediated by hydrogen peroxide, which also acts as a hemolysin. *M. pneumoniae* stimulates B and T lymphocytes and induces formation of autoantibodies which react with a variety of host tissues and the I antigen on erythrocytes, which is responsible for production of cold agglutinins [2]. Recently, an ADP-ribosylating toxin with limited sequence homology to the pertussis toxin S1 subunit was described. The toxin is now known as the community-acquired respiratory distress syndrome toxin (CARDS TX). It causes vacuolation and ciliostasis in cultured host cells [20] and may prove to be a significant virulence factor in *M. pneumoniae.* Although mycoplasmas are generally considered to be extracellular organisms, intracellular localization is now appreciated for *M. pneumoniae* as well as other mycoplasma species including *M. fermentans, M. penetrans* and *M. genitalium,* [21, 22]. Intracellular localization may be responsible for protecting the organisms from antibodies and antibiotics, as well as contributing to disease chronicity and difficulty in cultivation in some cases.

The genome of M. *pneumoniae* M129, the type strain for *M. pneumoniae* group I was sequenced in 1996. It consists of 816 kbp with 687 protein coding genes [23]. The 811 kbp genome sequence of the FH strain, which is the type strain for the *M. pneumoniae* group II was published in 2010 [24]. The G+C content for both genomes is ~39%, which is more than 15% higher than for any other known mollicute. *M. pneumoniae* appears in electron micrographs as pleomorphic rods 0.1–0.2 μm in width with a prominent attachment organelle [3]. This mycoplasma can be cultivated at 37°C in 5% CO_2 in air in SP4 medium containing glucose at pH 7.4–7.6. Spherical colonies develop after several days.

16.2.2 Genus Ureaplasma

The genus *Ureaplasma,* established in 1974, [25] comprises those members of the family *Mycoplasmataceae* that hydrolyze urea and use it as a metabolic substrate for generation of ATP. Unlike the genus *Mycoplasma*, ureaplasmas lack hexokinase and arginine deiminase, so they cannot utilize glucose or arginine. Shepard provided the first description of ureaplasmas, initially known as "T-strain" mycoplasmas, when he cultivated them *in vitro* from the urethras of men with nongonococcal urethritis (NGU) [26]. This genus currently has seven recognized species that have been isolated from humans and various animals*: U. canigenitalium* (dogs), *U. cati* (cats), *U. diversum* (cattle), *U. felinum* (cats), *U. gallorale* (chickens), *U. parvum* (humans), and *U. urealyticum* (humans). Numerous other ureaplasmas of animal origin have been described, but they have not been given species designations.

Human ureaplasmas were originally considered to belong to a single species, *U. urealyticum,* until 2002 when its two biovars were reclassified as the separate species *U. parvum* and *U. urealyticum*, based on the sequences of 16S rRNA gene, the 16S–23S-rRNA intergenic region, the urease gene and DNA–DNA hybridization experiments [27]. There are 14 known serovars distributed between the two species that

were initially identified by comparing reactions of human and animal antisera with clinical isolates as well as assays with polyclonal or monoclonal antibodies directed against whole cells or purified antigens. *U. parvum* contains the serotypes 1, 3, 6, and 14 while the remaining serovars 2, 4, 5, 7–13 are assigned to *U. urealyticum.*

As many as 40–80% of healthy adult women may harbor ureaplasmas in their cervix or vagina. The organisms are readily transmitted venereally as well as vertically. Their occurrence is somewhat less in the lower urogenital tract of healthy men (approximately 20–29%) [28, 29]. *U. parvum* is more common than *U. urealyticum* as a colonizer of the male and female urogenital tracts and in the neonatal respiratory tract [6]. Ureaplasmas reside primarily on the mucosal surfaces of the urogenital tracts of adults or the respiratory tracts in infants. Despite their frequent occurrence in the lower urogenital tracts of healthy persons, *Ureaplasma* spp. may cause or be associated with a variety of clinical conditions including urethritis, arthritis, chorioamnionitis, postpartum endometritis, preterm birth and pneumonia, bacteremia, abscesses, meningitis and chronic lung disease in preterm infants [6].

Ureaplasmas are capable of attaching to a variety of cell types such as urethral epithelial cells, spermatozoa, and erythrocytes [6]. The adhesins of ureaplasmas have not been characterized completely, but current evidence suggests the receptors are sialyl residues and/or sulfated compounds [6]. A major family of surface proteins, the multiple banded antigens (MBA), is immunogenic during ureaplasmal infections. MBAs have been used as a basis for the development of reagents for diagnostic purposes [28–31], However, recent work shows MBA expression to be phase variable, so false negatives using assays based on MBA protein or mba gene detection are possible [32, 33]. Ureaplasmas produce an IgA protease and release ammonia through urea hydrolysis, both of which are considered possible virulence factors [6]. An intact humoral immune response appears to be important in limiting invasion and dissemination of ureaplasma beyond mucosal surfaces. This is demonstrated by their tendency to cause chronic respiratory infections and arthritis in persons with hypogammaglobulinemia, and to cause invasive disease in preterm neonates [6]. Variation in surface antigens of *Ureaplasma* spp. may be related to persistence of these organisms at invasive sites.

Ureaplasma genomes range in size from approximately 750 kbp (average 608 genes) for *U. parvum* to approximately 950 kbp (average 664 genes) for *U. urealyticum.* The % G+C of DNA is 25–27. These organisms typically appear as coccoid cells of about 0.2–0.3 μm diameter under electron microscopy, but may be as small as 0.1 μm [34, 35]. *Ureaplasma* spp. grow rapidly in appropriate media containing urea such as 10B broth and A8 agar and will produce colonies 15–60 μm colonies within 1–2 days after incubation at 37°C in air plus 5% CO_2. Colonies appear brown and granular in the presence of a $CaCl_2$ indicator in A 8 agar and may produce the fried egg effect. Ureaplasmas require an acidic pH (6.0–6.5) for optimum growth [1]. The appearance of brown granular colonies on A8 agar is sufficient for the diagnosis of *Ureaplasma* spp. in a clinical specimen, but culture alone cannot distinguish between the two species.

16.3 Detection of Mollicutes by Traditional Methods

16.3.1 Culture

Culture is a quick and reliable method for detection of *Ureaplasma* spp. and *M. hominis* in clinical specimens. These organisms will produce color change in broth and colonies will develop on agar within 1–3 days if suitable growth media and incubation conditions are provided. Culture has an additional advantage in that it provides an isolate that can be studied further, and on which antimicrobial susceptibility testing can be performed if needed. However, for *M. fermentans, M. pneumoniae*, and especially *M. genitalium*, culture is seldom used since it is an insensitive method for detection and may require several days to weeks for evidence of growth to become apparent. The high failure rate of culture, even in experienced reference laboratories mandates that alternative methods such as PCR-based assays must be used. Detailed methods for obtaining specimens and culturing mycoplasmas and ureaplasmas of humans *in vitro* have been described in other reference texts [1, 34].

16.3.1.1 Phenotypic Identification of Mollicutes Grown in Culture

For *Ureaplasma* spp., the appearance of granular colonies on A8 agar in the presence of $CaCl_2$ indicator is sufficient for genus identification. Species detection will require additional molecular-based assays as described in subsequent sections. However, for routine diagnostic purposes, identification to the genus level is sufficient. Unfortunately, the growth of a large-colony mycoplasma from a genital specimen cannot be conclusively identified to species level based on phenotypic appearance or biochemical activities. *M. hominis* is the most common mycoplasmal species isolated from urogenital or extragenital cultures. If color change occurs in 10B or SP4 broth containing arginine within 1–3 days and colonies with a fried egg appearance occur on A8 or SP4 agar within a similar time frame, the organism is most likely *M. hominis* [1]. However, to ensure accuracy of results, it is necessary to perform additional confirmatory testing to exclude the possibility one of the commensal mycoplasmal species may be present. The same situation applies to a respiratory specimen cultured for *M. pneumoniae,* even though culture is not widely used for routine diagnostic purposes. Growth of a glycolytic mycoplasma after several days of incubation is most likely *M. pneumoniae*, but this must be confirmed by immunological or molecular-based assays.

Prior to the development of PCR-based assays in the 1990s, there were several alternative methods used to identify mycoplasmas detected by culture in clinical specimens. One method that has been used to identify suspected *M. pneumoniae* colonies from respiratory specimens is the hemabsorption test. This technique involves overlaying mycoplasmal colonies with guinea pig erythrocytes, washing and examining microscopically for attached erythrocytes covering the colonies. A second method for identification of *M. pneumoniae* is to test for its capacity to

reduce the colorless compound triphenyl tetrazolium to the red compound formazan. Other mycoplasmas from the human respiratory tract do not hemabsorb or reduce tetrazolium. A third method for speciation that has also been used for *M. hominis* is epi-immunofluorescence. This technique involves flooding an agar plate containing mycoplasmal colonies with species-specific antibodies conjugated to fluorescein isothiocyanate that is then washed and examined under a fluorescent microscope. The main limitations of this procedure are that antisera are not widely available for most of the human mycoplasmal species, cross-reactivity often occurs, the tests are poorly reproducible and often difficult to interpret due to background fluorescence. A fourth method that has been used to speciate large-colony mycoplasmas is the agar growth inhibition technique. This method involves application of filter paper disks impregnated with species-specific antisera onto agar plates onto which the unknown mycoplasma has been inoculated. Plates are then incubated with the anticipation that growth of the corresponding species will be inhibited. Like the epi-immunofluorescence method, this technique is cumbersome to perform, has many limitations, and requires rigorous attention to positive and negative controls. Several different antisera may be required to encompass multiple strains within a given species and some species that are antigenically similar such as *M. pneumoniae* and *M. genitalium* are likely to show cross-reactions. Immunoblotting with monoclonal antibodies, metabolism inhibition tests, and mycoplasmacidal assays have also been used to identify mycoplasmas in previous years. Fortunately, the development of PCR assays has resulted in much less reliance on the tests described above, which were never very satisfactory.

16.3.2 Serological Detection

16.3.2.1 *M. pneumoniae*

Serological testing was the first method for detection of *M. pneumoniae* infections, and was used almost exclusively for many years until the development of PCR assays reduced the need somewhat. Despite its widespread use and general acceptance among clinicians, serology has far more limitations than advantages for detection of acute *M. pneumoniae* infections. The original complement fixation tests have been replaced by commercial assays in a variety of formats including enzyme immunoassay (EIA), particle agglutination, and immunofluorescence. The main disadvantages of serology are the need for both acute and convalescent paired sera collected 2–3 weeks apart that are tested simultaneously for IgM and IgG to confirm seroconversion, difficulty in distinguishing current or recent infection from past infection, and the need to wait 1–2 weeks from the day of infection until detectable antibody develops. This is especially important in adults over 40 years of age who may not mount an IgM response, presumably because of reinfection [2]. Moreover, IgM antibodies can sometimes persist for several weeks to months, making it risky to base diagnosis of acute infection on a single assay for IgM [2]. Antibody

production may also be delayed in some infections, or even absent if the patient is immunosuppressed. False-negative tests for IgM can also occur if serum is collected too soon after onset of illness. Serological testing of *M. pneumoniae* and the various commercial methods available for antibody measurement have been described in other reference texts and reviews and will not be discussed further here [1, 2, 34].

16.3.2.2 Urogenital *Mycoplasma* and *Ureaplasma* spp.

Serological tests for *M. hominis* and *Ureaplasma* spp. using the techniques of microimmunofluorescence, metabolism inhibition, and EIA have been described [36–39]. A microimmunofluorescence assay for *M. genitalium* has also been developed [40] and shown to detect antibody responses in men with urethritis [41] and women with salpingitis [42]. A sensitive and specific serological assay for *M. genitalium* using lipid-associated membrane proteins (LAMP) as antigens has also been developed and this technique has been used in combination with Western immunoblots to assess immunoreactivity of women who were regarded as culture-positive for *M. genitalium* [43]. Despite investigations over several years aimed at developing serological assays for genital mycoplasma and ureaplasma infections, ubiquity of these organisms in healthy persons makes interpretation of antibody titers difficult and the mere existence of antibodies alone cannot reliably distinguish among colonization, current, or prior infection [1]. Rapid growth in culture for *M. hominis* and *Ureaplasma* spp. and dependence on PCR assays for detection of *M. genitalium* have effectively limited serological assays for these genital infections to research tools. The assays described above have never been standardized and are not generally available or recommended for diagnostic purposes.

16.4 Molecular-Based Detection, Identification, and Genotyping

Interest in non-culture-based detection of mycoplasmas in clinical specimens dates back to the 1980s when ELISA kits for direct antigen detection and non-amplified DNA probes were developed for detection of *M. pneumoniae* in respiratory specimens. However, the analytical sensitivity of these methods was poor and this hindered their acceptance. Once highly sensitive nucleic acid amplification tests (NAAT) began to be developed, interest in non-amplified DNA probes and antigen detection assays waned and there are no products of this nature sold commercially in the United States for detection of mycoplasmas.

Over the past two decades hundreds of publications have appeared in the literature describing various NAATs and their applications to detect mycoplasmas and ureaplasmas in clinical specimens since the first one in 1989 in which a conventional PCR based on the ATPase operon was described for *M. pneumoniae* [44]. These assays enable detection of extremely fastidious species such as *M. genitalium*

that might never be detected otherwise, and identification of organisms to the species level as in the case of ureaplasmas. PCR is the most widely applied NAAT for detection of mycoplasmas and ureaplasmas in clinical specimens. Many different target genes have been used as described in subsequent sections. PCR has also been adapted to detect antimicrobial resistance determinants and to analyze genetic relatedness of clinical isolates. Conventional PCR measures the end stage PCR products using gels or other methods while real-time PCR detects and quantifies the products simultaneously with amplification. Nested PCR may increase sensitivity 100-fold through re-amplification of a PCR product with a second set of primers [45]. However, the nested PCR method may also enhance the risk of contamination [46]. Real-time PCR is capable of amplifying and simultaneously detecting and quantifying the target DNA molecule as it accumulates during the reaction in *real time* after each amplification cycle. Detection systems include agarose gel electrophoresis, SYBR green, TaqMan probes, hybridization probes, molecular beacons, and microchip electrophoresis [47]. Other NAATs applied to mollicutes include nucleic acid sequence-based amplification (NASBA) and loop-mediated isothermal amplification (LAMP). Since organism viability does not have to be maintained for NAAT-based detection, specimen collection, handling, and transport is much simpler than for culture. Moreover, NAAT is inherently more sensitive than culture, when optimum conditions and gene targets are employed, making it an attractive alternative in many clinical and research settings. While conventional PCR methods can take 2–3 days, real-time PCR can potentially provide results the same day a specimen is received and provide quantitative data to determine the bacterial load in a clinical specimen. This can be important for interpretation of results for organisms such as *M. pneumoniae* that are known to colonize asymptomatic persons.

Genetic variability among bacterial species has been studied with a variety of approaches aimed at examination of antigenic or genomic profiles. Some techniques have proven to be better than others for organisms such as mollicutes with very small genomes. PCR and selective or whole genome sequencing are now popular methods for characterization of mollicutes and determining genetic relatedness, but other molecular-based methods have provided useful information before PCR-based methods were widely available. These include immunoblotting with monoclonal antibodies, 2-dimensional gel electrophoresis or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), pulsed field gel electrophoresis (PFGE), DNA–DNA hybridization, and restriction endonuclease cleavage. All of these methods have been used to assess variability in antigenic profiles and/or genomes among the human mycoplasmal and ureaplasmal species over the past several years and the general conclusion of most studies has been that these organisms exhibit a great deal of heterogeneity as a result of antigenic variation and gene transfer within and among species.

The following sections summarizes general information regarding PCR, other types of NAATs and older non-amplified methods as they have been developed and applied to detect and characterize mycoplasmas and ureaplasmas of humans for research or diagnostic purposes. There is considerably more experience with these types of assays for some species, such as *M. pneumoniae,* than for others. In some instances different typing methods have been compared with one another to

evaluate their discrimination capacity for a specific organism. With respect to application of PCR methods for diagnostic purposes, the concept of a genus-level PCR to detect any organism within the genus *Mycoplasma* may be useful in some cases.

16.4.1 Mycoplasma spp.

16.4.1.1 *M. fermentans*

Considerable controversy exists concerning the putative role of *M. fermentans* as an agent of human disease. Difficulty in cultivation of this mycoplasma in vitro led to development of PCR assays for its detection in clinical specimens. The most common PCR targets for this mycoplasma have been the 16S rRNA gene [48, 49], although other targets such as insertion sequence IS1550, and *malp* (macrophage activating lipopeptide gene) [50] have been used.

The UAB Diagnostic Mycoplasma Laboratory has adapted and validated a real-time PCR procedure to detect *M. fermentans* originally developed by Blanchard [49] for conventional PCR using the 16S rRNA gene target, the Roche LightCycler 2.0 (Roche Diagnostics, Indianapolis, IN) and *M. fermentans* PG-18 (ATCC #19989) as the positive control. Primers from the 16S rRNA gene are: RNAF1 (10 μM): 5′CAGTCGATAATTTCAAATACTC-3′ and RNAF2 (10 μM): 5′ GGCACCGTCAAAACAAAAT-3′.

Though *M. fermentans* has not been studied to the extent as other *Mycoplasma* species from humans, a few studies have attempted to characterize genetically a small numbers of clinical isolates [51–55]. Studies of this nature are extremely limited due to the fact that this mycoplasma is isolated so infrequently in clinical specimens.

Schaeverbeke genotyped seven strains isolated from synovial fluids of seven arthritis patients in an attempt to determine whether they were unique strains of the same organism and compared them to three reference strains (PG18, K7, and *incognitus*) and a clinical isolate from the urogenital tract using arbitrarily primed PCR, conventional restriction enzyme analysis, PFGE, and Southern blotting [54]. Overall, the varied means used in this study to characterize the genomes of these *M. fermentans* clinical isolates showed that two groups were represented among them. Four synovial fluid isolates were genetically related to the reference strain PG-18 while the remaining ones and the urethral isolate were related to reference strain K7 and *incognitus*.

Another study by Campo [55] examined 21 *M. fermentans* strains from varied geographic and patient sources using PCR, Southern blotting and DNA hybridization, SDS PAGE and Western blotting with monoclonal antibodies. This study focused on the distribution and characteristics of the insertion sequence (IS) element. They determined that there were two genotypes represented, which differed with respect to the sites of insertion of the IS element, but not in the sequence of the

element itself. They concluded that the method for species detection would be direct detection of sequences within the IS element, but for genotyping within the species, PR primers RS47 and RS49 could be used for genotype I and RS 47 and MF-4 for genotype II eliminating the need for cultivation.

To our knowledge, there have been no reports of genetic characterization of *M. fermentans* using PCR-based analyses that do not require organisms cultivated *in vitro*. The findings of a few studies indicate heterogeneity of *M. fermentans* and support the need for larger studies to include additional isolates or molecular-based typing that can be applied directly to clinical specimens. The recently published genome sequence for *M. fermentans* may also facilitate design of better PCR assays for this species [12].

16.4.1.2 *M. genitalium*

In comparison to *M pneumoniae* and *M. hominis,* the data on *M. genitalium* with regards to epidemiology, disease associations, and laboratory methods for detection and characterization are somewhat less, largely due to its relatively more recent discovery in the early 1980s, its somewhat infrequent occurrence in the urogenital tract of adults, and the demanding cultivation requirements which have led to very few isolates being available for study. Lack of reliable cultivation techniques has necessitated intense study of molecular-based techniques for detection and characterization.

Even though *M. pneumoniae* and *M. genitalium* are structurally and even antigenically related, they are genomically different with *M. genitalium* being considerably smaller. As was the case for *M pneumoniae,* a DNA probe for *M genitalium* was developed in the 1980s, but was eventually proven to lack sufficient sensitivity to be clinically useful. Attention shifted to PCR in the early 1990s [56, 57] with development of assays that amplified various fragments of the MgPa adhesin protein gene derived from the original G37 type strain that could detect as few as ten organisms. At least nine PCR assays have targeted various regions of the MgPa operon as shown in Table 16.3. Other reports soon followed with a variety of formats for PCR assays using this gene target and the 16S rRNA gene [58, 59]. Additional molecular-based assays including transcription-mediated amplification (TMA) have also been described and utilized for epidemiological purposes [60–62]. As with other mollicutes, quantitative, rapid, real-time PCR assays have been developed that allow measurement of bacterial load in clinical specimens [62–68] using targets such as MgPa, 16S rRNA, and the *gap* gene encoding glyceraldehyde-3-phosphate dehydrogenase. As experience increased with a variety of assay formats for molecular-based detection of *M. genitalium* in clinical specimens, it became apparent that not all primers utilized in various studies reacted with all *M. genitalium* strains, especially those using the MgPa target. Ma and colleagues [69] recently examined the three genes of the *M. genitalium* MgPa operon (*mgpA, mgpB and mgpC*) and nine repetitive sequences MgPars dispersed throughout the genome in 15 geographically diverse strains to investigate their sequence variability and its

Table 16.3 Diagnostic PCR assays targeting the MgPa Operon of *Mycoplasma genitalium*

Forward primer (5′-3′)	Reverse primer (5′-3′)	Reference
MgPa-1, AGTTGATGAAACCTTAACCCCTTGG	MGpA-3, CCGTTGAGGGGTTTTCCATTTTTGC	[57]
Mg1 (outer) TGTCTATGACCAGTATGTAC Mg3 (inner) GTAATTAGTTACTAGTAGA	Mg2, CTGCTTTGGTCAAGACATCA	[56]
MgPa-476, ATGGCGAGCCTATCTTTGATCCTTTTAA	MgPa-903, TTACCTCCCCACTACTGTCCTTATGC	[184]
G3A, GCTTTAAACCTGGTAACCAGATTGACT	G3B, GAGCGTTAGAGATCCCTGTTCTGTTA	[185]
MGS-1, GAGCCTTTCTAACCGCTGC	MGS-4, GTTGTTATCATACCTTCTGAT MGS-2, GTGGGGTTGAAGGATGATTG	[186]
Mg1a, GGTTAACTTACCTTAGTGGCTTTGATC Mg3 (inner), GTAATTAGTTACTCAGTAGA	MGS-2, GTGGGGTTGAAGGATGATTG	[187]
ModMgPa1, TGAAACCTTAACCCCTTGG	ModMgPa3, AGGGGTTTTCCATTTTTGC	[188]
MgPaW1, AATGGAGCGATCATTACTAAC	MgPaWR1, CCTTGTTATCATACCTTCTGA	[189]
MgPa-355F, GAGAAATACCTTGATGGTCAGCAA	MgPa-432R, GTTAATATCATATAAAGCTCTACCGTTGTTATC	[68]

potential implication for both pathogenesis and development of diagnostic tools. They discovered that all operon sequences as well as all MgPars differed from each other more than from the published G37 operon sequence at both the nucleotide and deduced amino acid levels. By mapping 19 previously published PCR primers used in diagnostic assays, these authors determined that 1 of 19 primers contained up to 19 variable nucleotides and that the target for one of two typing systems was located in a hypervariable region, indicating the likelihood of erroneous results with some of the assays. This has been demonstrated in studies using primers MGS-2 and MgPa-903 [69]. Taken together, these data suggest that there is an efficient recombination system enabling generation of numerous variants that may facilitate evasion of host defenses and that additional research and development must be undertaken to identify and validate appropriate PCR primers for diagnostic testing.

In view of concerns for use of the MgPa target relating to the variations among *M. genitalium* strains, and 98% identity of the 16S rRNA gene for *M. pneumoniae* and *M. genitalium,* the UAB Diagnostic Mycoplasma Laboratory has adapted the real-time PCR assay described by Svenstrup [66] that was shown to be both sensitive and specific for detection of *M. genitalium* in clinical specimens. This protocol targets the conserved housekeeping gene *gap* (National Center for Biotechnology Information accession no. U39710) in a primer and probe system. This target is different from other species, including the *gap* homologue in *M. pneumoniae* (72.3% identity) and is present in the genome as a single copy [66]. *M. genitalium* strain G-37 (ATCC #33530) is used as the positive control. The forward primers (10 μM) are mg-gap-605f: 5′-GTGCTCGTGCTGCAGCTGT-3′ and reverse primers (10 μM) are mg-gap-794r: 5′-GTCCATCTGTTGAACAAGTAAATCAAGC-3′. Probes (4 μM) consist of a fluorescein-labeled probe are mg-gap-669FL: 5′-TGTTGTTCCAGAAG CAAATGGCAAACTT-FL-3′ and (4 μM) Red640 probe mg-gap-700LC: 5′-LCRed640-GGGATGTCACTCCGTGTTCCAGTGT-phosphate-3′.

PCR-based assays have also been developed to detect mutations in DNA gyrase and/or topoisomerase IV mediating fluoroquinolone resistance in *M. genitalium,* thereby circumventing the need for culture in vitro to determine antimicrobial susceptibilities to these agents [70]. A major limitation in the use of NAATS for detection of *M. genitalium* in clinical specimens is lack of commercially sold tests in the United States. Gen Probe (San Diego, CA) has developed a TMA real-time PCR assay that performs well in comparison to other methods, but it is available in the United States only for research purposes [62, 71, 72]. Development of commercial PCR assays for detection of *M. genitalium* and other human urogenital mycoplasmas and ureaplasmas in clinical specimens from the urogenital tract are much further advanced in Europe since the requirements there are not so stringent as those mandated in the United States by the Food and Drug Administration. Consequently, many products of this nature have not been subjected to large and rigorous clinical trials or comparisons with existing assays with publication in peer-reviewed scientific journals. Multiplex PCR-based systems for detection of *M. genitalium* along with *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, or other urogenital mycoplasmas and ureaplasmas are sold as kits in several European countries by multiple companies including Bio-Rad (Hercules, CA), Amplex Biosystems

(Giessen, Germany), PCR Diagnostics.eu (Bratislava, Slovak Republic), and Seegene (Rockville, MD) using various formats and instrument platforms.

Kokotovic et al. provided the first description of a typing method for *M. genitalium* strains based on whole genome fingerprinting involving selective amplification of restriction fragments obtained from purified DNA of cultured strains [73]. However, further attempts to develop genotyping methods for the examination of clinical isolates have been very limited since the organism is so difficult to cultivate *in vitro* and only a few strains are actually available worldwide. In recent years, typing methods that are not dependent on having a cultivated clinical isolate have been developed in order to overcome this limitation. Molecular typing methods used for *M. genitalium* are based on PCR amplification of a specific genomic locus followed by DNA sequencing or restriction fragment length polymorphisms (RFLP) analysis. Specific methods have included short tandem repeat (STR) analysis of putative liproprotein gene MG309 [74], single nucleotide polymorphisms (SNPs) in the rRNA operon [74], RFLP of the *mgpC* gene [75], and SNPs in the *mgpB* gene [64, 76].

In one study [76], a PCR assay based on the *mgp* gene of the MgPa operon using the MgPa-1/MgPa-3 primer set was applied to urogenital specimens from patients in multiple countries. This method identified 29 different sequences from 52 unrelated patients and also demonstrated that 79 *M. genitalium*-positive specimens from 19 couples had concordant sequence types, indicative of sexual transmission. They were also able to demonstrate acquisition of new strains concordantly in sexually active couples. The occurrence of a few large clusters indicated either spread of certain strains or particularly common sequence types that were detected in specimens from different countries. These findings indicate the heterogeneity of *M. genitalium* isolates from clinical specimens.

Multilocus variable number tandem repeats (MLVA) is a relatively new molecular typing method based on the variation in the copy number of tandemly repeated sequences (VNTRs) located in different genetic loci. Ma and colleagues [77] used MLVA to identify 18 loci in the G37 *M. genitalium* reference strain containing STRs and determined that combination of *mgpB* SNPs and MG 309 STRs complement one another, thereby providing greater typing efficiency, and may define genetic relationships more accurately. Addition of MG307 STRs and MG338-STRs is potentially useful to study sexual transmission of *M. genitalium* infections. However, recent evidence suggests that the use of the *mgpC* gene for RFLP may be unsuitable for genotyping since this region of the genome is undergoing rapid sequence shifts due to homologous recombination with MgPars repeats [77].

16.4.1.3 *M. hominis*

M. hominis grows rapidly in culture so development of PCR assays for detection has not been as important as for other species such as *M. pneumoniae* and *M. genitalium.* However, the many advantages of PCR have driven investigations to develop assays for diagnostic and research purposes. Conventional PCR assays for *M. hominis* have mainly utilized 16 S rRNA and rDNA as gene targets [78, 79].

Since some heterogeneity has been reported in the 16 S rRNA gene of *M. hominis* [80], other targets including the *yidC* gene targeting a membrane protein translocate have been considered [81]. The UAB Diagnostic Mycoplasma Laboratory utilizes a real-time PCR assay to detect *M. hominis* in clinical specimens using the *gap* gene (National Center for Biotechnology Information accession no. AJ243692). This assay using the Roche LightCycler 2.0 (Roche Diagnostics) was adapted from the original report of Baczynska [82]. *M. hominis* PG-21 (ATCC #23114) is used as the positive control. The following primers and probes are used in volumes of 1 μL each: MHGAPF (10 μM): 5′-GGAAGATATGTAACAAAAGAAGGTGCTG-3′; MHGAPR (10 μM): 5′-TTTATCTTCTGGCGTAATGATATCTTCG-3′; MHGAP 336FL (4 μM): 5′- AGCAGGTGCTAAAAAGGTGTTTATTACTGCTCC - FL-3′; MHGAP 370LC (4 μM): 5′- LC Red 705-GCTAAAAGCGAAGGTGTTAAAA CAGTTGTTTATTCAGTA-3′.

Genetic variability among different strains of *M. hominis* has been studied by several approaches directed towards examination of antigen profiles and genomic sequences. Ladefoged and Christiansen [83] constructed physical and genetic maps of the genomes of five *M. hominis* strains obtained from different individuals. Genome sizes obtained by PFGE ranged from 704 to 825 kb. Restriction patterns varied greatly and none of the strains had identical restriction patterns with any of the five restriction endonucleases used. Other methods including serology, DNA–DNA hybridization, 2D gel electrophoresis, SDS-PAGE, restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), and immunoblotting with monoclonal antibodies have also confirmed heterogeneity within the species [84–87]. Blanchard [88] used conventional PCR to evaluate genetic variation within the 16S rRNA gene of 51 *M. hominis* isolates from the urogenital tract of men and women which were initially identified using immunoblotting with monoclonal antibodies. The DNA–DNA hybridization values ranged from 51 to 100%, even though 16S rRNA genes showed only minor strain to strain variation, confirming high intraspecies conservation within this gene. However, 16S rRNA gene sequencing also documented differences at five positions when one isolate was compared to the PG21 reference strain. Jensen [84] studied 60 *M. hominis* isolates from pregnant women and their offspring using SDS-PAGE and PFGE and determined that sequential isolates from the same women were identical or nearly identical, suggesting adaptation to the host environment, whereas those from different women exhibited considerable variation with respect to both genomic and antigenic profiles.

16.4.1.4 *M. pneumoniae*

Among the mycoplasmas that colonize and infect humans, *M. pneumoniae* is generally considered the most important pathogen and it is logically the species for which the greatest efforts have been made to develop and refine molecular tools for its detection and characterization. Gene targets NAATs have included 16S rRNA, 16S rDNA, the P1 adhesin gene, the *tuf* gene, *parE* gene, *dnak* gene, *pdhA* gene, ATPase operon, CARDS toxin gene, and the noncoding repetitive element *repMp1*

[45, 47, 89–92]. Analytical sensitivity of these assays is generally high, with some of them capable of detecting a single organism when purified DNA is used.

The UAB Diagnostic Mycoplasma Laboratory has adapted the real-time PCR assay published by Dumke [91] targeting the *repMp1* noncoding DNA sequence for routine diagnostic use. Its theoretical advantage is the fact that sensitivity may be improved by amplification of a multicopy gene [91]. The following primers are used: 0.80 μl of 10 μM MpLCrepF: 5′ TCTTTACGCGTTACGTATTC-3′ and 1.0 μl of 10 μM MpCrepR: AGTGTGGAATTCTCTGGCA-3′. The probe consists of 1.0 μl of 2 μM MpLCrepS: 5′FAM-CTGGTATAACCGGTTTGTTAAG-TAMRA-3′. This assay provides acceptable sensitivity and specificity when tested against several reference strains as well as a large group of specimens from patients with radiologically proven pneumonias, representing both major P1 subtypes, who were positive for *M. pneumoniae* by serology, culture, and or conventional PCR. Utilization of PCR for detection of *M. pneumoniae* infection in extrapulmonary sites, such as cerebrospinal fluid in a patient with neurologic illness, is also helpful since cultures from non-pulmonary sites are very rarely positive. PCR has also been shown to be advantageous for diagnostic purposes when insufficient time has elapsed since onset of illness for an antibody response to develop and for testing preserved lung tissue obtained at biopsy [89]. The advantage of real-time PCR over conventional PCR in detection of systemic infection was demonstrated in a study which found 15 of 29 (52%) patients with serological evidence of *M. pneumoniae* infection had a positive assay while conventional PCR was uniformly negative [93].

While PCR may theoretically be more sensitive than culture-based detection, some studies with *M. pneumoniae* have shown this is not the case [89]. For PCR-positive, culture-negative patients, it is important to ascertain whether clinically significant respiratory disease is actually present, since this may reflect asymptomatic carriage with a very low bacterial load, prior antibiotic therapy, persistence of mycoplasmal DNA after resolution of infection has occurred, organisms residing in an intracellular location not amenable to culture, or perhaps a nonspecific PCR target. A positive PCR assay in a patient who is serologically negative may indicate that the specimen was obtained too early in the infection for measurable antibody to have developed, antimicrobial therapy that may have blunted the immune response, or an inadequate immune response due to any type of immunosuppressive condition. Negative PCR results in patients who are culturally and/or serologically positive could indicate technical problems with the PCR assay or inhibitors. Sometimes employing a PCR assay utilizing a different gene target may resolve the problem. One issue that is sometimes apparent with PCR assays for *M. pneumoniae* is that they are not tested adequately for specificity for *M. pneumoniae* by making certain there is no reactivity with the numerous commensal mycoplasmal species of the human respiratory tract.

Performing PCR assays with one or possibly two gene targets is generally the best diagnostic approach for *M. pneumoniae* infections but using the second target increases costs. Combining PCR with serology has been advocated as a possible means to distinguish colonization from active disease, but this also adds to the cost of testing and will not overcome the problem common in older adults who do not

mount an acute phase IgM serological response in many instances, necessitating testing paired sera and prolonging the time until diagnosis can be confirmed.

There is no universal consensus regarding what constitutes the best respiratory specimen to be tested by PCR. Combining nasopharyngeal and oropharyngeal specimens may provide the greatest diagnostic yield [94]. Another study reported that sputa was superior to oropharyngeal or nasopharyngeal specimens in young adults with serologically proven *M. pneumoniae* infection [95]. From a practical standpoint in sampling young children and many adults with fairly mild illness, sputum is not produced, so nasopharyngeal or oropharyngeal samples may be the only specimen types available.

A significant concern that has been raised with regards to noncommercial *M. pneumoniae* PCR tests that are used for diagnostic purposes in the United States is that there have been very few side-by-side comparisons to determine whether one assay format or gene target is better than another. Many of the comparisons that have been done compared PCR with culture or serology as mentioned above and, as might be expected, got disparate results in some cases. A study from the Centers for Disease Control and Prevention [90] has addressed this matter to some extent by comparing three real-time PCR assays to detect *M. pneumoniae* prospectively in an outbreak investigation. They performed triplicate PCR assays utilizing the Applied Biosystems ABI 7500 system employing two different TaqMan primer-probe sets targeting the ATPase gene and a new assay targeting the CARDS toxin gene on 54 respiratory samples from an outbreak in a college setting. Primers/probes for the CARDS toxin were: M181-F TTTGGTAGCTGGTTACGGGAAT; M181-R GGTCGGCAC GAATTTCCATATAAG; M181-P TGTACCAGAGCACCCCAGAAGG GCT. Eighteen cases were positive with all three assays. When dilutions of *M. pneumoniae* reference strains were tested, the CARDS toxin PCR assay consistently detected 1–5 CFU while the other two assays targeting the ATPase genes detected 5–50 CFU. These findings support further study of the CARDS toxin gene as a PCR target. There have been two published studies describing multicenter comparisons of various NAATs for *M. pneumoniae* detection [96, 97]. Both studies reported significant variations in test performance among participating laboratories, making a strong case for an organized proficiency test program, which has been pilot-tested in Europe and is now used in some molecular diagnostic laboratories [98].

Multiplex PCR assays have been developed in a variety of formats for detection of *M. pneumoniae* along with other respiratory pathogens that may produce clinically similar illnesses such as *Chlamydophila pneumoniae* and *Legionella pneumophila* [99–102]. A few assay formats now detect more than 20 pathogens and some of them are developed as commercial assays. Generally speaking, some loss in analytical sensitivity occurs in multiplex assays when compared to monoplex assays that may be related to incompatible amplification conditions for multiple targets and the high concentration of primers that can cause elevated background readings and reduced efficiency. Few multiplex assays have been tested on a large scale in a clinical setting or compared rigorously to other methods of detection so it is not possible to judge how good they really are. Multiplex assays for detection of *M. pneumoniae* and other respiratory pathogens have also been combined with other techniques

including reverse line blot assays and microarrays [103–105]. These techniques are discussed in more detail in the subsequent section on ureaplasmas.

Although PCR is the most common means of molecular-based diagnosis of acute *M. pneumoniae* infections, the expensive equipment necessary can be an obstacle to its utilization in many hospitals. One DNA amplification method that does not require a thermocycler is the LAMP assay. This technique amplifies DNA under isothermal conditions with high efficiency, specificity, and speed and has been applied to detection of *M. pneumoniae* in clinical specimens using the P1 gene sequences for primer development in direct comparison to real-time PCR using the Roche LightCycler with the P1 gene target [106]. They showed this assay to be specific with a detection limit of 200 copies and found 100% concordance with real-time PCR when applied to 95 nasopharyngeal specimens.

NASBA is another molecular diagnostic method that can be used to amplify RNA sequences in both conventional and real-time formats. Real-time NASBA uses DNA hybridization probe molecular beacons that fluoresce only upon hybridization with their targets. They have a stem-loop structure and contain a fluorophore and a quencher group. In its normal state, the stem keeps the fluorophore and the quencher together, preventing emission of fluorescence. In the presence of a sequence that is complementary to the loop sequence, the probe unfolds upon hybridization, the quencher no longer absorbs photons emitted by the fluorophore, and the probe starts to fluoresce. The whole process of amplification and detection takes place in a fluorescence reader [107]. NASBA can provide rapid results with a sensitivity as good or better than PCR, with reports of a detection threshold as low as 5–50 CFU for *M. pneumoniae* [107, 108]. The main advantage of NASBA is that it works at isothermal conditions, so it does not require a thermocycler instrument. This assay has been described in monoplex and multiplex format and has been developed as a commercial kit (NucliSens, bioMérieux) targeted at RNA in a real-time system using fluorescent DNA hybridization probes. While the multiplex NASBA assay has potential to detect *M. pneumoniae* as well as *C. pneumoniae* and *Legionella* spp., it had a slightly lower sensitivity than monoplex NASBA when applied to dilutions of wild-type in vitro generated RNA [109].

Commercial PCR assays, including monoplex and multiplex systems, have been available in Europe for several years and some are still in development. Limited evaluations for some products have shown they work in a comparable manner to noncommercial assays [101, 102, 110]. Some of these assays include the Artus RepMp1 (Qiagen), Venor Mp-QP (Minerva Biolabs), Chamylege (Argene Inc.), and Pneumoplex (Prodesse Inc.). As of 2012, there are no commercial PCR assays for *M. pneumoniae* sold in the United States.

A comprehensive and up to date review of diagnostic methods for acute *M. pneumoniae* respiratory infection that includes an in depth discussion of various NAAT methods, including real-time PCR assays was recently published by Loens and coworkers [47]. They commented that as of 2010 there were at least 61 published in-house PCR assays for *M. pneumoniae,* many of which have been validated only for their analytical sensitivity and not tested against large numbers of clinical samples or against one another. The reader is referred there for details on the many

Table 16.4 Examples of molecular-based assays and gene targets used for detection of *Mycoplasma pneumoniae* published since 2005

Gene target	Assay type	Detection format	Mono or multiplex	Reference
P1	PCR	Real-time	Monoplex	[190]
P1	LAMP	Turbidimiter	Monoplex	[106]
P1	PCR	Scorpion probe	Monoplex	[191]
P1	PCR	Hybridization	Multiplex	[102]
repMp1 in P1	PCR	Real-time	Monoplex	[91]
CARDS toxin	PCR	Real-time	Monoplex	[90]
ATPase	PCR	Real-time	Monoplex	[90]
tuf	Broad-range PCR	Real-time	Monoplex	[192]
16S rRNA	PCR	Real-time	Multiplex	[101]
16S rRNA	PCR	Molecular beacons	Monoplex	[193]
16S rRNA	PCR	Real-time	Monoplex	[100]
Not Specified	PCR	Resequencing microarray	Multiplex	[194]

LAMP loop-mediated isothermal amplification

NAATs developed for *M. pneumoniae.* A summary of some of the more recently described NAAT assay formats for detection of *M. pneumoniae* that have been included in published articles is provided in Table 16.4.

Among the mycoplasma species isolated from humans, *M. pneumoniae* has been the most extensively studied species with regards to its genomic and protein profiles as a means to improve understanding of its epidemiology and pathogenicity. The most significant work in molecular genotyping of *M. pneumoniae* has been done over the past two decades and was made possible by the characterization and sequencing of the 170 -kDa P1 gene [111, 112]. Although the genetic sequences of the P1 protein genes differ among *M. pneumoniae* strains, they provide the basis for development of most genotyping assays. Initial studies included Southern blot analyses of genomic DNA using subclones of the P1 gene as probes to establish that clinical isolates can be classified into two P1-subtypes based on type-specific repetitive elements [112, 113]. This classification was later verified by restriction enzyme fingerprinting and two dimensional gel electrophoresis [114]. As molecular-based technologies advanced over the past several years, conventional and real-time PCR and other NAAT-based typing procedures such as NASBA, multilocus sequence typing (MLST), and MLVA have been employed by investigators in several different countries to characterize clinical isolates of *M. pneumoniae* [115–128]. The general finding has been that *M. pneumoniae* is a fairly uniform organism, despite different geographical origins.

M. pneumoniae tends to occur endemically with epidemics every few years [2]. Observations in Denmark over a several year period in which a high number of cases occurred between epidemics without a return to lower endemic levels led to speculation that differences in the circulating strains may account for such changes since some degree of natural immunity lasting a few years may occur after acute *M. pneumoniae* infection [129, 130]. Studies from Japan [122, 127] and Germany [131] have shown that differences in the two main P1 adhesin subtypes may be

operative in the development and cycling of epidemics. Those investigators found that one or the other subtype tended to predominate in specific geographic areas and that there were changes in the predominance of one or the other subtype over time, but this is not always the case according to other studies in which both subtypes may occur in about the same proportions [125]. This predominance of one or another subtype may be due to development of subtype-specific antibodies following initial infection that provide some degree of protection for that subtype but not the other. Dumke reported differences between the P1 subtypes in their capacity to colonize and survive in a guinea pig model and that pre-infection of the animals with the different subtypes induced subtype-specific immunity and affected the type of surviving bacteria [128]. However, Ursi found no relationship between subtype and degree of illness in 24 patients [132]. Clearly, additional work needs to be done in this area to ascertain whether there is a relationship between P1 subtypes and virulence.

Dorigo-Zetsma [120] carried the subclassification further using RFLP analysis of PCR products of the P1 gene as previously described [118], but with six additional restriction enzymes, sequencing of 16–23S rRNA gene spacer regions and PCR-RFLP of large inter-repeat fragments of DNA. They identified 5 subtypes within the P1 type 1 group and an additional 3 subtypes within the P1 type 2 group among a collection of 2 reference strains and 21 clinical isolates. The clinical or epidemiological significance of this extended typing scheme has not been determined.

While considerable knowledge has been gained over a several year period as a result of numerous PCR-based methods of *M. pneumoniae* strain subtyping, the relative homogeneity of this mycoplasma limits discrimination by PCR-RFLP of the P1 gene. PFGE of *M. pneumoniae* strains allows study of the whole genome as opposed to a single gene [118] and enables division of P1 subtype 2 into two additional subgroups, but as discussed in more detail in the subsequent section on ureaplasmas, it is a very time-consuming and tedious procedure to perform. Multilocus sequence typing (MLST) is widely used to type many bacterial pathogens, but an attempt to use this method with structural and housekeeping genes for typing *M. pneumoniae* with its small genome and limited polymorphism among housekeeping genes did not prove it to be a valuable means for molecular typing [116].

MLVA is another whole-genome approach that has been utilized for genotyping *M. pneumoniae* by Dégrange et al. [125] by selecting variable number tandem repeats (VNTRs) from the sequence of strain M129 for which the complete genome sequence was published [23] to type 265 isolates from various countries. VNTR markers were selected using Tandem Repeats finder software and the Microorganisms Tandem Repeats database (http://minisatellites.u-psud.fr/). Ultimately five VNTR loci were selected for utilization. Two multiplex PCRs with fluorescently labeled primers to amplify the five loci. Data were analyzed using GeneScan and GeneMapper software version 4 (Applied Biosystems). Data with the calculated number of repeats were imported into Bionumerics software, version 5 (Applied Maths) and a minimum sparing tree (MST) was generated and a polymorphism index of individual or combined VNTR loci was calculated. The five loci were efficiently amplified from all 265 strains revealing 26 VNTR types. Each type was closely

related, differing by no more than one locus without any distinct clusters. The MLVA technique greatly expands the typing scheme for *M. pneumoniae* beyond what is offered by the other comparative method of PCR-RFLP that can only identify the two major P1 types and variants, but there was a correlation detected between the two methods. Since this study was performed using endemic strains and none were collected during an epidemic it is unclear what different findings would be in that setting. A particularly attractive feature of this typing method is that it could potentially be adapted to direct testing of clinical specimens without requirements for the bacterial isolates, thereby greatly expanding its application in surveillance and epidemiology of *M. pneumoniae* infections.

As described earlier with *M. genitalium,* advances in molecular detection methods has enabled culture-independent methods for genotyping, which is particularly advantageous when there are relatively few clinical isolates of the organism of interest available. Dumke applied a nested PCR-based assay to *M. pneumoniae* DNA isolated from 108 respiratory tract specimens in which part of the *repMp2/3* element of the P1 gene was amplified followed by sequencing. The product of the first amplification was then subjected to a semi-nested PCR with primers Mp5f an Mp19r to amplify the complete repetitive element *repMp2/3* analyzed with an ABI Prism 377 DNA sequencer (Applied Biosystems) [121]. They were able to classify 75% of the specimens into one of the two P1 subtypes, with the remainder belonging to 2 variants. This new approach for molecular typing of *M. pneumoniae* is important since it can theoretically expand epidemiological studies as well as identify emergence of new combinations of repetitive elements in the P1 gene that may occur through homologous recombination.

Macrolide resistance in *M. pneumoniae* is becoming a major problem in Asia and is now spreading to Europe and North America [89]. Since culture is time consuming and insensitive, alternative methods for identifying resistant strains was needed. Investigators in Europe and the United States have developed real-time PCR assays to detect three major mutations in domain V of 23S rRNA that confer high level macrolide resistance in isolates of *M. pneumoniae* or directly in clinical specimens [133–135]. This method of direct detection of resistance genes is based on the fact that nucleic acid will melt at a precise temperature that is related to the nucleotide base composition. The presence of one or more point mutations in 23S rRNA that impair antimicrobial agent attachment to the bacterial ribosome will be detected by this extremely sensitive method, which can be completed in just a few hours. A rapid and inexpensive method that combines nested PCR, single-strand conformation polymorphisms (SSCPs), and capillary electrophoresis (CE) detects macrolide-resistant mutants directly from throat swabs [136]. Pyrosequencing technology has also been applied for detection of macrolide resistance in *M. pneumoniae* as well as for molecular strain typing [137].

The UAB Diagnostic Mycoplasma Laboratory performs a multiplex real-time PCR assay to detect point mutations in all three positions of the 23S rRNA gene as shown in Fig. 16.2. This assay uses FRET (Fluorescence Resonant Energy Transfer) hybridization probes and the Roche LightCycler 2.0 instrument. The detection limit is as low as 7 mutant molecules/μl in the PCR mixture. This assay, described in detail in a previous

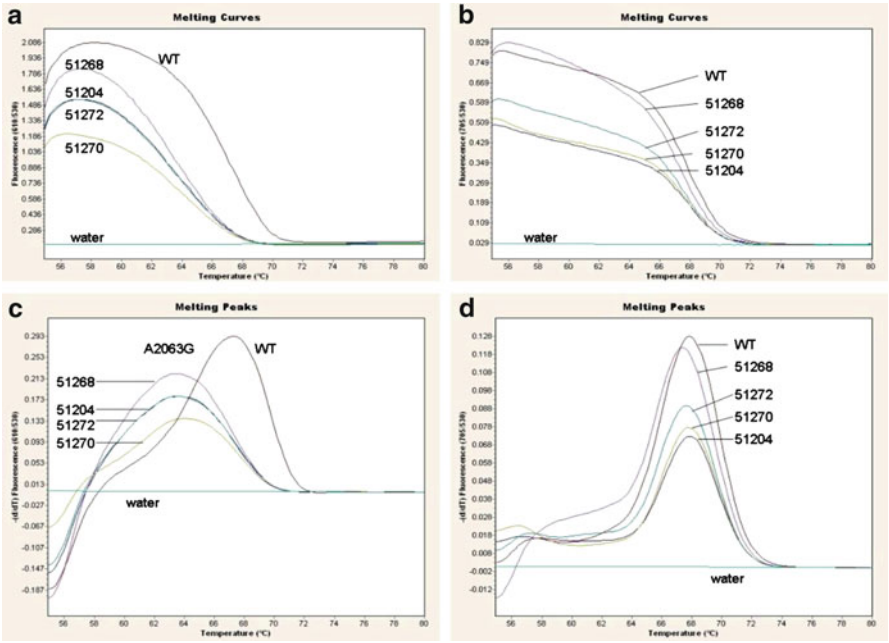

Fig. 16.2 Real-time PCR Detection of Macrolide-resistant *M. pneumoniae* in Clinical Specimens. Genomic DNA two patients containing the A2063G mutation verified by sequencing were purified and tested together with a wild type (WT) control (*M. pneumoniae* strain M129, ATCC #29342). Melting curves (1a, 1b) and corresponding melting peaks (1c, 1d) are shown. A2063/A2064 mutations were analyzed in channel 610 (1a, 1c). The WT melting peak was 67.31°C, while Tm of A2063G mutants were 63.25 ± 0.04°C. Thus a 4°C difference between WT and mutant was observed. The C2617 assay is shown in channel 705 (1b, 1d). Because all samples did not have mutations at this position, they showed similar WT Tms of about 68°C as predicted. Adapted from reference 134 and reproduced with permission from the Pediatric Infections Disease Journal (Wolfers Kluwer Health) [134]

publication [134], is performed reflexively whenever there is a positive real-time PCR assay for the presence of the organism. For the detection of macrolide resistance mutations, primers MpnMR2063F and MpnMR2063R as shown in Table 16.5 define a 224 bp amplicon containing the 2063/2064 position where mutations are likely to occur that is recognized by probes MpnMR2063P1 and MpnMR2063P2.

Several French [125], German [138], Japanese [139, 140], and Chinese [141–144] investigators have examined macrolide-resistant *M. pneumoniae* in an attempt to ascertain how these infections have evolved and spread geographically. The isolates were molecularly typed either by PCR- RFLP of the P1 gene or by MLVA. No clear association was observed between the macrolide-resistant isolates and the P1 subtypes, while the more discriminating MLVA analysis on French and Japanese isolates did not reveal any link between a particular MLVA type and macrolide resistance [125]. These data confirmed the absence of a particular emerging macrolide-resistant clone spreading from country to country.

Table 16.5 Primers and probes used in real-time PCR for primary detection and identification of macrolide resistance in *M. pneumoniae*[a]

Primers	
MpLCrepF	5′- TCTTTACGCGTTACGTATTC-3′
MpLCrepR	5′- AGTGTGGAATTCTCTGGAA -3′
Probe	
MpLCrepS	5′- FAM-CTGGTATAACCGGTTTGTTAAG-TAMRA-3′
Secondary assay performed on *M. pneumoniae* positive specimens to detect 23S rRNA mutations	
Primers	
MpnMR2063F	5′-ATCTCTTGACTGTCTCGGC-3′
MpnMR2063R	5′-CCAATTAGAACAGCACACAACC-3′
MpnMR2617F	5′-GGCTGTTCGCCGATTAAAG-3′
MpnMR2617R	5′-TACAACTGGAGCATAAGAGGTG-3′
Probes	
MpnMR2063P1	5′-GGCGCAACGGGACGGAAAGA-Fluorescein-3′
MpnMR2063P2	5′-LC Red 610-CCGTGAAGCTTTACTGTAGCTTAATATTGATCAGG-Phosphate-3′
MpnMR2617P1	5′-GTCCCTATCTATTGTGCCCGTAGGAAG-Fluorescein-3′
MpnMR2617P2	5′-LC Red 705-TGAAGAGTGTTGCTTCTAGTACGAGAGGACCGAA-Phosphate-3′

[a]Detection method uses Roche LightCycler with the repMp1 gene target originally described by Dumke [91] for primary detection and the method of Li Xiao [134] for identification of 23S rRNA mutations associated with macrolide resistance

The advent of a new generation of DNA sequencing technology will enable biologists to sequence many different *M. pneumoniae* clinical isolates with minimal effort and expense. As these genomic data become available it will become possible to more effectively design PCR and other NAAT assays that will be more capable of providing useful diagnostic results about the nature of and probability for acute disease inherent in different strains of *M. pneumoniae.*

16.4.2 Ureaplasma spp.

As with *M. hominis*, the rapid growth of ureaplasmas in culture has meant that molecular-based detection is not essential for routine diagnostic work, but it is important to identify the individual species and for research purposes. Gel-based conventional PCR assays developed for detection of *Ureaplasma* spp. have targeted sequences of 16S rRNA gene and 16S rRNA -23S rRNA intergenic spacer regions [145–149] the urease gene, [150, 151] and the *mba* genes [28, 148, 152–155]. Published real-time PCR assays have targeted primarily the urease gene and its subunits [156–158] and the *mba* gene [29, 159]. Yoshida and coworkers [79] described a conventional PCR-based method that can be applied to urine specimens

of patients with NGU. The PCR targets are the 16S rRNA genes of *M. genitalium, M. hominis, U. parvum*, and *U. urealyticum.* Following amplification, PCR products were then subjected to hybridization assays in a 96 well microtiter plate using four species-specific capture probes to detect the targets. When compared to direct sequencing, this technique produced similar results and showed no cross reactivity. Its advantages include less cost and a 2–3 h turnaround time.

The oligonucleotide primers for specific *Ureaplasma* gene targets sometimes behave differently when used with the various thermocyclers, protocols, and reaction mixtures. Thus, each primer set needs to be customized for the instrument to be used just as they would need to be for other organisms. Primer development software for the respective instruments is useful to guide this process.

The UAB Diagnostic Mycoplasma Laboratory in conjunction with scientists at the J. Craig Venter Institute have recently developed and validated a real-time PCR assay for detection and simultaneous speciation of ureaplasmas in human infections that is now used for diagnostic purposes [29]. The assay is based on the *UU063* gene (NP_077893), which encodes a conserved hypothetical protein that is identical in all 4 *U. parvum* serovars and a 15,072 bp open reading frame (ORF), UUR10_0680, that is conserved (>99.97%) in all 10 *U. urealyticum* serovars. Details of this procedure are provided in Table 16.6 as example of how real-time PCR using the LightCycler 2.0 can be applied to detect these organisms as well as *Mycoplasma* spp. This assay, which detects ureaplasmas in clinical specimens and designates which of the two species is/are present, compared favorably with culture and actually detected more positive clinical specimens than a conventional PCR assay based on a urease gene target.

Some molecular-based assays that includes detection of *Ureaplasma* spp. in clinical specimens are commercially available in various European countries, but not in the United States at present. Seegene, Inc. (Rockville, MD, USA, markets their products STD6 and STD6B ACE Detection that simultaneously detects *Trichomonas vaginalis, M. hominis, M. genitalium, C. trachomatis, N. gonorrhoeae*, and *U. urealyticum* in endocervical/urethral swabs. The novel feature of the Seegene technology is a dual priming oligonucleotide system that contains two separate priming regions linked by a polydeoxyinosine spacer [160]. The polydeoxyinosine has no binding specificity or priming, but instead forms a bubble or tiny D-loop that results in more stable priming. The Seegene STD6 ACE kit works with any thermocycler and the post-PCR assay is designed for either manual or automated gel electrophoresis. The STD6 *Ureaplasma* assay is not species-specific. It amplifies a 130 bp region of the *ureD* gene cassette [161]. The new version of their kit, STD6B, for detecting urogenital pathogens differentiates *U. urealyticum* from *U. parvum* by targeting the *U. urealyticum ureD* and *U. parvum ureC* genes. As mentioned earlier in the discussion for *M. genitalium,* other companies including PCR Diagnostics.eu and Amplex Biosystems also sell PCR-based diagnostic products to detect *Ureaplasma* spp. in clinical specimens. PCR Diagnostics.eu sells an assay in traditional PCR format with detection of PCR products on agarose gels that will differentiate the two *Ureaplasma* species.

Table 16.6 Real time PCR procedure for detection of *Ureaplasma* species in clinical specimens

1. Set up PCR in a biosafety cabinet that has been cleaned with 70% ethanol or 10% chlorine bleach. Equipment needed includes (1) pipettes: a set of 2, 20, 200 and 1,000 μl pipettes, wipe before and after using with 70% ethanol or 10% chlorine bleach; (2) corresponding pipette tips; (3) LightCycler PCR capillaries (20 μl); (4) LightCycler Centrifuge Adaptor (precooled at 4°C). Put the glass capillaries into the adaptor; (5) Precooled (4°C) racks (Benchtop freezer); (6) Small Biohazard waste bag
2. Prepare a work sheet. Calculate total reaction numbers and reagent volumes. Each sample is run in duplicate. Include single reaction with external control
3. Turn on the computer, LightCycler 2.0 and LightCycler Carousel Centrifuge. Open LightCycler Software 4 and setup programs. Name and save a new file for each run
4. Run self-test
5. Prepare Master Mix as shown below. Allow frozen reagents to thaw on ice. Add all ingredients together in the listed sequence. Mix well by pipetting up and down, spin down for 5 s. Aliquot 16 μl to all PCR capillaries
6. Add 4 μl of PCR-grade H_2O to the negative control capillary and cap it
7. Add 2 μl of PCR-grade H_2O to all the other capillaries except those for internal (positive) controls
8. Add 2 μl of clinical sample DNA to the corresponding capillary. Discard tips in the Biohazard waste bag. Cap all of the triplicate-reaction capillaries. Check pipette and change gloves frequently
9. Add 2 μl of internal (positive) control to the corresponding capillary and cap it. This consists of a 1:10 mixture of *U. parvum* serovar 3 (ATCC 27815) and *U. urealyticum* serovar 8 (ATCC 27618). Change gloves when switching form patient samples to positive controls, or vice versa
10. Insert the capillaries into the corresponding holes of PCR carousel. Centrifuge the carousel using the LightCycler Carousel Centrifuge 2.0
11. Make certain all the liquid sinks to the bottom of the capillary, then put the carousel into the LightCycler 2.0 and close the lid
12. Click Start Run to begin the PCR program
13. Generate color compensation file in a separate run for each new lot of oligonucleotides to compensate cross-talk between individual channels must be compensated using a color compensation object in a separate run. Use Master Mix for color compensation file as described below. Cycle the samples with the PCR program. When finished, select Color Compensation from the Analysis menus and click Save CC Object
14. After PCR is completed, use LCS4 software to analyze results. First, perform Absolute Quantification analysis. When probes annealed to target DNA, fluorescences (640 nm or 705 nm) emitted by fluorescence resonance transfer (FRET) from donor probes to the acceptor probes (UP063#1 Probe1 to UP063#1 Probe2 or UU127#1 Probe1 to UU127#1 Probe2) are measured by channel 640/back530 (for *U. parvum*) and channel 705/back530 (for *U. urealyticum*). Apply valid Color Compensation file to the analysis. Check the cross-point (C_p) value of each PCR sample. Positive control should show a C_p value around 30. Negative control should not show a C_p value. Then, do Tm Calling analysis. Settings are set as high sensitive and 2 peaks or less. Positive control and samples with internal control should show similar Tm values, which are 67.56 ± 0.51°C for *U. parvum* serovar 3 and 65.72 ± 0.30°C for *U. urealyticum* serovar 10. Positive samples should also show similar Tms as positive controls. Negative control and samples should not have Tm values in this range. An inhibited PCR sample is determined by unable amplification of internal control in the clinical sample (no C_p value)

Master Mix and PCR Program for Detection of *U. parvum* and *U. urealyticum*

PCR-grade H_2O	3.65 μl
25 mM $MgCl_2$	1.60 μl
Primer1: UP063#1F (10 μM)	0.60 μl
Primer 2: UP063#1R (10 μM)	1.00 μl
Primer 3: UU127#1F (10 μM)	0.40 μl
Primer 4: UU127#1R (10 μM)	1.00 μl
Probe1: UP063#1 probe 1 (4 μM)	0.75 μl
Probe2: UP063#1 probe 2 (4 μM)	0.75 μl
Probe3: UU127#1 probe 1 (4 μM)	1.00 μl
Probe4: UU127#1 probe 2 (4 μM)	1.00 μl
LightCycler Uracil-DNA Glycosylase (2 U/μl)	0.25 μl
5× LightCycler Multiplex DNA Master HybProbe	4.00 μl
Total volume	16 μl

Program name	Cycles	Analysis mode	Target (°C)	Hold (hh:mm:ss)	Slope (°C/s)	Sec target (°C/s)	Step size	Step delay (cycles)	Acquisition mode
Pre-incubation	1	None	40	00:10:00	20	0	0	0	None
			95	00:10:00	20	0	0	0	None
Amplification	50	Quantification	95	00:00:15	20	0	0	0	None
			55	00:00:10	20	0	0	0	Single
			72	00:00:09	20	0	0	0	None
Melting	1	Melting Curves	95	00:00:00	20	0	0	0	None
			60	00:00:30	20	0	0	0	None
			95	00:00:00	0.1	0	0	0	Continuous
Cooling	1	None	40	00:00:30	20	0	0	0	None

(continued)

Table 16.6 (continued)

Master Mix for Color Compensation Object (total 15 µl)	
PCR-grade H_2O	6.15 µl
25 mM $MgCl_2$	1.60 µl
Primer1: UP063#1F (10 µM)	0.60 µl
Primer 2: UP063#1R (10 µM)	1.00 µl
Primer 3: UU127#1F (10 µM)	0.40 µl
Primer 4: UU127#1R (10 µM)	1.00 µl
LightCycler Uracil-DNA Glycosylase (2 U/µl)	0.25 µl
5× LightCycler Multiplex DNA Master HybProbe	4.00 µl
Total volume	15 µl

Multiply the amount by the number of reactions (×5). Pipet 15 µl of Master Mix to the capillary. Add additional water and probes to the corresponding capillary

Capillary	Water (µl)	Probe (µl)
Blank	5.00	0
Probes	4.00	0.50 µl UP063#1 probe 1; 0.5 µl UU127#1 probe 1
LC-Red 640 probe		5.00
LC-Red 705 probe		5.00

Primers and Probes for *Ureaplasma* PCR:

UP063#1F: 5′-TGCGGTGTTTGTGAACT-3′
UP063#1R: 5′- TGATCAAACTGATATCGCAATTATAGA -3′
UU127#1F: 5′- CAGTAGCAAATCGTGCTTACA-3′
UU127#R: 5′- TCATTAAAATCATTTGCACTAGTCAAAATA-3′
UP063#1 Probe1: 5′-TGG-TTT-AAC-GTG-TTT-TTG-AAG-TGC-TAC-AAA-AT-Fluorescein-3′
UP063#1 Probe2: 5′- LC Red 640-CCC-ATT-TCA-GCC-ATG-GTG-CCA-TCA-Phosphate -3′
UU127#1 Probe1: 5′-GAT-AAT-AAC-ACT-TGG-ACA-ATT-TTT-AAC-CAA-AGC-GA-Fluorescein
UU127#1 Probe2: 5′-LC Red 705-AAG-GAT-TAG-AGT-TTT-GTT-GCC-ATG-GTA-GTC-AAA-Phosphate-3′

PCR can also be used in combination with other techniques including reverse line hybridization blotting combined with multiplex PCR (mPCR/RLB) and microarrays [103, 104, 162, 163]. The mPCR/RLB method requires a visualization step based on specific interaction between biotin and conjugated-streptavidin. The mPCR/RLB technique integrates multiplex PCR (mPCR) using biotin-labeled primer pairs or biotin-dNTPs to generate labeled PCR products that hybridize with highly specific, membrane-bound, amine-labeled oligonucleotide probes by reverse line blot hybridization. To visualize the hybridized PCR products, the membrane is incubated with peroxidase-labeled streptavidin and chemiluminescent substrate, e.g., electrochemiluminescence (ECL) detection liquid. Chemiluminescence results can be detected by a light sensitive film or a lumino-imager [163]. The mPCR/RLB technique has been used to develop multiplex assays that detect numerous respiratory pathogens including *M. pneumoniae* and urogenital pathogens, including both *Ureaplasma* spp., *M. genitalium*, and *M. hominis* [103, 105, 164]. The microarray used for the detection of *Ureaplasma* spp. and some other microorganisms is based on immobilizing probes on a glass surface that hybridize to their complimentary biotin labeled-DNA targets produced by a previous PCR procedure. The microarray is then incubated with gold-conjugated streptavidin. The interaction of biotin and streptavidin leads to silver precipitation onto the streptavidin-bound nanogold particles. This is visualized as black spots on the microarray [165].

In situ hybridization (ISH) is a non-PCR-based molecular technique that has been specifically utilized for detection of ureaplasmas. ISH allows visualization of the localized gene expression within the context of tissue morphology. This methodology has been used for detection of *Ureaplasma* in lung tissue samples collected from intranasally inoculated newborn mice. After fixation of the tissue samples, the slides were incubated with biotinylated DNA probes specific for an internal nucleotide sequence within the urease gene. The hybridization signal was amplified by binding of peroxidase-conjugated streptavidin to biotin and biotinyl tyramide incubations. A chromogenic dye producing a brown precipitate at the hybridization sites was used to visualize the hybridization signal [166].

It has been speculated for many years that some individual *Ureaplasma* species or serovars might be associated with certain diseases more than others. Several studies reported that *U. urealyticum* is more pathogenic than *U. parvum,* but conflicting results have been found by others [6]. It has been considered that differential pathogenicity might exist at the serovar level rather than at the species level, necessitating an accurate means to distinguish among the 14 serotypes to address this question.

The methodologies initially developed to classify *Ureaplasma* isolates to species and serovar levels consisted of antibody-based phenotyping methods such as growth/metabolism inhibition tests, antibody-linked epi-immunofluorescence or color reaction. These methods yielded inconclusive results because of multiple cross-reactions and poor discriminating capacity. Moreover, reagents to perform these assays have never been widely available outside of the laboratory where they were developed. Molecular genotyping methods are more rapid and accurate, readily separating the two *Ureaplasma* species [146, 151, 156–158].

Published PCR assays to characterize the *Ureaplasma* spp. at the serovar level have mainly focused on primers based on the *mba* gene and its 5′-end upstream regions. In combination with direct sequencing or restriction enzyme analysis, these assays were capable of distinguishing the 4 serovars of *U. parvum* and dividing the 10 serovars of *U. urealyticum* into different subgroups [28, 153, 154, 159, 167]. Because of limited sequence variation in the *mba* genes, earlier PCR-based methods lacked the capacity for complete separation of all 14 serovars. Moreover, whole genome sequencing of all 14 serovars has shown *mba* to be part of a large gene family present in many variations in different serovars and the gene is phase variable [29]. In consideration of the limitations of earlier serotyping techniques, a 14 serovar-specific monoplex PCR assay was developed and shown to accurately distinguish between the two species and among all 14 serovars without cross-reactivities [29]. Data from examination of large numbers of clinical isolates in the culture collections of the UAB Diagnostic Mycoplasma Laboratory using this serovar-specific PCR assay have shown that *Ureaplasma* pathogenicity is unlikely to be associated with specific individual serovars and that horizontal gene transfer among serovars results in many organisms expressing markers of multiple serovars simultaneously. These findings suggest that serotyping is impractical and of limited value for assessment of pathogenicity [168]. Primers and probes utilized to distinguish the 14 Ureaplasma serovar type strains are shown in Table 16.7.

The question remains as to whether significant variation exists within individual *Ureaplasma* serovars that could account for differential pathogenicity. PFGE is a widely accepted reference standard for genotyping bacteria suitable to address this unresolved issue. In *Ureaplasma* spp., PFGE was applied initially to determine genome size. More recently, PFGE was used to differentiate *U. parvum* from *U. urealyticum*, to distinguish among the 14 serovars, and clinical isolates of the same serovars [169, 170]. In order to improve understanding of the differential pathogenictiy and characterize the genetic relatedness of *Ureaplasma* isolates within the same serovar, we used a simplified PFGE method to analyze *Ureaplasma* isolates of different origin that had been typed to species and serovar level by the species and serovar-specific real-time PCR assays described above.

A simplified PFGE method for *Ureaplasma* based on the procedures described previously [169, 170] has been developed. This method shortens the time needed for completion of the assay by 2–3 days while producing similar results. To perform PFGE, a frozen pellet of ureaplasma cells is thawed rapidly at 37°C. A volume of 4 μl of lysozyme (100 mg/ml, Sigma, St Louis, MO) and 10 μl of proteinase K (20 mg/ml, Sigma) is added to the cell suspension and equilibrated at 40°C. Cell-agarose mixtures (0.9%) are made by adding an equal volume of 1.8% agarose in TE buffer with 1% of *N*-lauroylsarcosine (Sigma). Plugs are formed and quickly lysed by incubating at 55°C for 90 min in proK-lysis buffer (6 mM Tris, pH 8.0, 1 M NaCl, 100 mM EDTA, 0.5% Brij-58 (Sigma), 0.2% sodium deoxycholate, 0.5% *N*-lauroylsarcosine, 1 mg/ml lysozyme, and 0.1 mg/ml proteinase K). After two washes in deionized water and another two washes in TE buffer at 50°C, plugs, can

Table 16.7 Primers and probes used in real-time PCR assays to detect and differentiate *Ureaplasma* spp.[a]

Specificity	Target gene/region	Primer pair	Probe	Reference
Up and Uu	16S rRNA	Ure-1S, Ure-1A	Ure-P1	[181]
Up	Urease gene subunits and adjacent regions	UPure F-M, Upure R-M	UPure1MGB	[158]
Up	Urease gene subunits and adjacent regions	UU-1524R, UU-1613F	UU-parvo	[157]
Up	*UU063*	UU063#1F, UU063#1R	UP063#1 Probe1, UP063#1 Probe2	[29]
Uu	*mba* gene and upstream regions	UU F, UU R		[156]
Uu	Urease gene subunits and adjacent regions	Uuure F-C, Uuure R-C	Uuure FP	[156]
Uu	Urease gene subunits and adjacent regions	Uuure F-M, Uuure R-M	Uuure2MGB	[158]
Uu	Urease gene subunits and adjacent regions	UU-1524R, UU-1613F	UU-T960	[157]
Uu	*UUR10_0554*	UU127#1F,UU127#1R	UU127#1 Probe 1, UU127#1 Probe 2	[29]
Serovar 1	*mba* gene and upstream regions	UP F, UP R	UP FP1	[159]
Serovar 1	Member of *mba* gene family but not *mba*	UP1F1-2, UP1R1-2	UP1 probe 1	[29]
Serovar 2	putative lipoprotein	UU2_F_1, UU2_R_1		[29]
Serovar 3	*mba* gene and upstream regions	UP F, UP R	UP FP3	[159]
Serovar 3	conserved hypothetical protein	UP3F1-2, UP3R1-2		[29]
Serovar 4	Intergenic	Uu04_1F, Uu04_1R	Uu04_1 probe 1, Uu04_1 probe 2	[29]
Serovar 5	ATP-dependent RNA helicase	Uu05-3F, Uu05-3R	Uu05-3 probe	[29]
Serovar 6	*mba* gene and upstream regions	UP F, UP R	UP FP6	[159]
Serovar 6	*mba*	UP6F1, UP6R1		[29]
Serovar 7	*mba*	UU7_F_1, UU7_R_1		[29]
Serovar 8	Intergenic	UU8_F_1, UU8_R_1		[29]
Serovar 9	FtsK/spoIIIE family protein gene	UU9_F_1, UU9_R_1		[29]
Serovar 10	Member of *mba* gene family but not *mba*	UU10_F_4, UU10_R_4	UU10_P_4	[29]
Serovar 11	Intergenic	UU11_F_1, UU11_R_1	UU11_P_1	[29]
Serovar 12	conserved hypothetical protein	Uu12_1F, Uu12_1R	Uu12_1 probe 1, Uu12 _1 probe 2	[29]
Serovar 13	conserved domain protein , intergenic region and putative single-strand binding protein	UU13_F_1, UU13_R_1		[29]
Serovar 14	*mba* gene and upstream regions	UP F, UP R	UP FP14	[159]
Serovar 14	Intergenic	UP14F1, UP14R1		[29]

[a]Actual primer sequences are not included due to space limitations but they can be found in the individually cited publications

be stored at 4°C or processed for restriction enzyme digestion. After equilibration of the plug slices in 1× restriction buffer for 15 min, 40 U *BssH* II or 30 U *BamH* I-HF restriction enzymes (both from New England BioLabs, Ipswich, MA) are added and the reactions are incubated at designated conditions for quick digestion (50°C, 2 h for *BssH* II and 37°C, 30 min for *BamH* I-HF). Digested agarose plug slices are positioned on an agarose gel (pulsed field certified agarose, Bio-Rad, Hercules, CA). PFGE is performed on a CHEF II Mapper (Bio-Rad, Hercules, CA) and conditions of 200 V (6 V/cm) and 14°C are used for all runs. For *BssH* II digested products, the running parameters were: 1.0% agarose gel, switch time 1–40 s, run for 20 h. For *BamH* I digested products, running condition A is used in the initial experiments for comparison of 14 ATCC type strains: 1.3% agarose gel, switch time 1–15 s, run for 20 h. Then running condition B is used for comparison of *U. urealyticum* serovars of clinical isolates and ATCC strains and consists of: 1.5% agarose gel, switch time 1–12 s, run for 28 h.

Gels are stained with ethidium bromide (0.5 mg/ml) for 30 min followed by destaining with deionized water for 30 min and gel images are then captured using a Bio-Rad GelDocXR. Each gel included MidRange I PFGE Marker to normalize patterns for analysis with BioNumerics (Applied Maths, Austin, TX). Dendrograms are produced using the unweighted pair group method with arithmetic averages based on Dice coefficients and band tolerance and optimization of 1%. A cutoff of ≥80% similarity of PFGE patterns is used to designate related isolates [171].

Using these modified PFGE methods, it is possible to separate readily the two *Ureaplasma* species from one another and most of the serovars, with the exception of some of the closely related serovars of *U. urealyticum* as shown in Fig. 16.3. Using PFGE to evaluate all 14 serovar type strains and a large number of clinical isolates distributed among the 14 serovars, we were able to show a wide genotypic heterogeneity in *U. parvum* and *U. urealyticum* type strains of different serovars as well as great genetic variation of strains within the same serovar for most of the 14 serovars. The method has been shown to yield consistent results when used to analyze strains that have been passaged in vitro multiple times. Thus, PFGE has the potential to discriminate and assess genetic relatedness of the *Ureaplasma* clinical isolates within the same species and even the same serovar. This degree of discrimination to the serovar level cannot be completely achieved by other molecular typing methods currently available which include PCR-based assays targeting single genes and intergenic regions [28, 29, 148, 172].

PFGE is based on comparing bacterial genomes for specific changes resulting the presence, absence, or relocation of hexanucleotide regions recognized by specific restriction enzymes. Overall, it is a very imprecise form of whole genome sequence analysis and cannot produce data with sufficient resolution to explain why one bacterial isolate causes disease and another does not. Whole genome sequencing does offer that kind of resolution; but DNA sequencing using Sanger chemistry is far too expensive and complex for routine clinical analysis. Next-generation DNA sequencing technologies such as 454 pyrosequencing or Illumina's sequencing by synthesis are on the verge of being comparable in price, speed, and requirements for starting

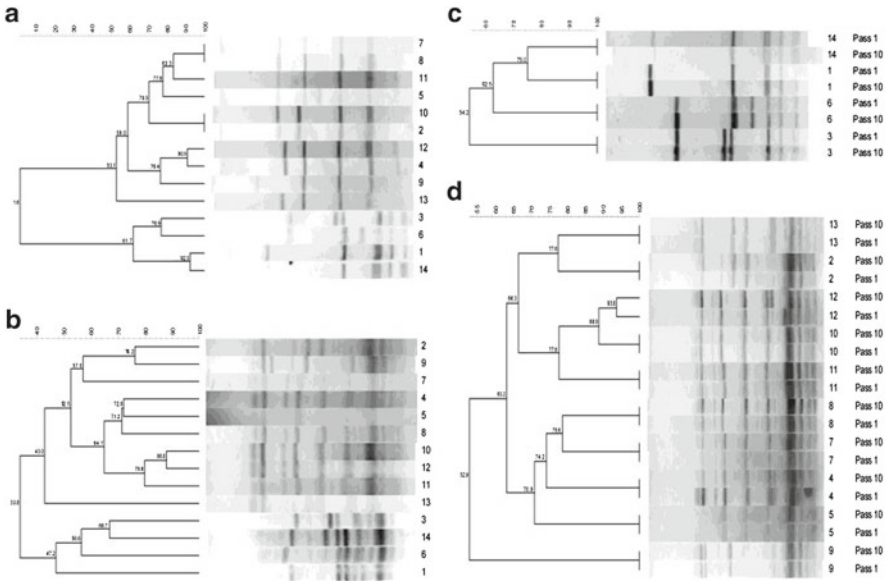

Fig. 16.3 PFGE banding patterns and dendrograms for 14 ATCC serovars of *Ureaplasma* spp. a) *BssH* II restriction pattern. *U. parvum* and *U. urealyticum* were separated in two major groups. Serovars were not well discriminated. b) *BamH* I restriction pattern. The two species were separated. Except for serovars 10 and 12, all of the serovars were also separated. c) Stability of *BssH* II banding patterns in *U. parvum* serovars after 10 serial passages. Patterns of pass 1 and pass 10 were identical and all 4 seroars were separated. d) Stability of *BamH 1* banding patterns in *U. urealyticum* serovars after 10 serial passages. Banding patterns of pass 1 and 10 of serovars 2, 4, 5, 7-9, 11-13 were identical

material to PFGE [173]. This is especially true for small genomes like those of ureaplasmas and mycoplasmas. Sequencing technology and the bioinformatics applications needed to cost-effectively analyze bacterial genomes in a manner that would be of clinical and epidemiological utility are on the horizon and will likely replace PFGE for these purposes.

The RAPD PCR method has been applied to *Ureaplasma* spp. by several investigators [172, 174–176]. It is quicker and less technically demanding than PFGE and is more discriminatory than the single-gene *mba* typing method [176]. However, there is no published study comparing PFGE and RAPD-PCR typing of *Ureaplasma* species. The MLVA typing method has not yet been developed and applied to *Ureaplasma* spp. One possible difficulty in method development for this technique may be related to the fact that there are very limited VNTR loci in ureaplasmas, perhaps due to extensive horizontal gene transfer.

16.5 Real-Time PCR Procedures for Detection of *Mycoplasma* and *Ureaplasma*

Publications describing real-time PCR for detection and characterization of mycoplasmas and ureaplasmas have utilized the ABI Prism 7900HT (Applied Biosystems, Carlsbad, CA); the iCycler iQ (Bio-Rad, Hercules, CA) and the LightCycler 2.0 (Roche Diagnostics, Indianapolis, IN). Over the past few years, the UAB Diagnostic Mycoplasma Laboratory has eliminated conventional PCR in favor of real-time PCR using the Roche LightCycler for diagnostic purposes because of its numerous advantages in accuracy and turnaround time. Therefore, detailed descriptions of procedures in this chapter are limited to real-time PCR assays using this platform. Other laboratories may choose to use one of the other acceptable real-time PCR instruments and methods.

The improved specificity of real-time PCR compared to conventional PCR is mainly due to the use of a third oligonucleotide probe used in many assays that binds to the target sequence. Usually, the probe is designed to correspond to a sequence located between the forward and reverse primers. Generally, when performing an assay with the LightCycler, the probe is labeled with a fluorophore and a quencher. When the quencher is in close proximity to the fluorophore, the fluorescence is inhibited or emitted at a different wavelength. Utilizing a fluorescently labeled probe allows a signal to be detected by the instrument only if the probe has annealed to its complementary target sequence and the DNA polymerase has amplified the target region therefore increasing the distance between the quencher and the fluorophore. The use of a labeled probe minimizes the probability of cross-reaction and detection of undesired amplicons. Such probes are also specifically useful when differentiating very closely related organisms is desired. Another feature of real-time PCR that contributes to the specificity of the assay is that the amplicon melting temperature is determined at the end of the assay, and can be used to verify whether the desired PCR product is being detected. Real-time PCR diagnostic assays are often designed to produce shorter amplicons with an optimal length of less than 300 bp compared to conventional PCR. The smaller the amplicon the more efficient is the PCR assay and the lower probability for the target templates to be sheared during handling.

16.5.1 Specimen Collection for Molecular-Based Assays

Any clinical specimens suitable for culture, including body fluids such as cerebrospinal fluid, peritoneal fluid, pleural fluid, semen, urine, and blood, nasal and endotracheal aspirates, urethral or cervical swabs, endometrial or lung tissues, bone, and the bacterial isolates themselves, are also suitable for diagnostic testing by molecular methods if they are collected, stored, and processed correctly. For best results, specimens to be analyzed by a PCR assay should be collected in sterile tubes or

vials with caps secured tightly to prevent leakage, frozen at −80 ºC within 1 h of collection, transported on dry ice to the laboratory where the test will be performed and remain frozen until DNA isolation. Blood (minimum 0.5 ml) should be collected in a vacutainer tube containing acid citrate dextrose (ACD). PCR can be performed directly on undiluted fluids. If an insufficient volume of original specimen necessitates dilution, culture transport media such as Shepard's 10B broth [177] or PBS (phosphate buffered saline, pH 7.4, without calcium and magnesium) buffer can be used. Specimens should be inoculated into 0.3–0.7 ml of transport media or PBS buffer at time of collection or as soon as possible thereafter. For swab specimens, use only Dacron or polyester swabs. Calcium alginate and cotton swabs can be inhibitory. Swab specimens should be rinsed in either culture transport media or PBS buffer and the swab extracted. Excess fluid should be removed from the swab by pressing the swab against the inside of the tube or cryovial before removing and discarding the swab. The 10B broth used for culture does not have any deleterious effect on performance of the real-time PCR assay we utilize with the Roche LightCycler, so it is suitable as a transport medium. However, it is possible that this culture broth could be inhibitory when using other primers and reaction conditions. Thus, it is mandatory to verify that culture broth is not inhibitory before using it for PCR transport. The advantage of using culture broth for PCR transport is that can be used interchangeably for specimen transport whether culture, PCR or both assays are to be performed on the same specimen. Use of transport medium is of greater importance for detection of organisms by culture than by PCR since viability of the organisms must be maintained for variable periods of time, depending on proximity of the laboratory to the clinical facility where specimens are obtained.

16.5.2 DNA Extraction

The simple lysis and proteinase K treatment usually yields PCR-detectable DNA unless the specimen is inhibitory [178]. Suitable specimens for this procedure include body fluids (other than blood) and transport media containing material obtained from swabs. This procedure can be performed as follows. First, chill a refrigerated bench top centrifuge to 4°C. Make certain two incubators have equilibrated to 60°C and 95°C, respectively. Label the tubes for sample processing. In the biosafety cabinet, thaw proteinase K lysis buffer (0.5 mg/ml, stored in −80°C freezer) and samples to room temperature. Once all the reagents and samples are thawed, keep them on ice. In the biosafety cabinet, vortex each sample and transfer the sample to the sample processing tube. Centrifuge at 14,000 × *g* for 20 min at 4°C. Make sure the tubes are oriented in a manner such that the pellet is easily observed. In the biosafety cabinet, discard the supernatant into the biohazard waste bag using a sterile filtered tip. Remove as much supernatant as possible. Add 200 µl proteinase K lysis buffer to the tube and vortex briefly at high speed to dissolve the pellet. Incubate at 60°C for 1 h then heat-inactivate the proteinase K by incubating samples at 95°C for 10 min. Store the deproteinized samples at −80°C until use.

Potentially inhibitory specimens including blood, tissue samples, lower respiratory secretions, and subcultures should be purified by the QIAamp® DNA Blood Mini Kit (Qiagen) or other commercial genomic DNA purification kits according to the manufacturer instructions. Tissues should be minced in 0.5 ml 10B broth or PBS buffer using sterile scissors prior to DNA extraction.

Automated or semi-automated nucleic acid isolation methods, such as Qiagen BioRobot EZ1 (Qiagen), nucliSens (bioMérieux), easyMAG (bioMérieux), or MagNaPure LC (Roche Applied Science), can also be used to prepare samples. Each laboratory must perform an evaluation of every assay component from sample type, transport media, extraction method, to final PCR amplification and detection procedures using the specific primers and probes, reaction conditions, and controls applicable to the assay to ensure the techniques are valid and there is no PCR inhibition at any step. Some automated extraction systems appear to work as well as manual systems. The easyMAG nucleic acid extractor actually enabled superior amplification results for *M. pneumoniae* when applied retrospectively to clinical specimens when compared to the QiaAmp blood mini kit and the NucliSens miniMAG systems [179, 180].

DNA preparation should be carried out in a biological safety cabinet, and separated from the PCR set up area. Fresh gloves must be worn when changing from patient specimens to controls and vice versa to avoid contamination. Dedicated filtered pipette tips for each processing area should be used in all PCR-related procedures. Clinical samples should be processed prior to processing controls.

16.5.3 LightCycler PCR Programs and Operating Conditions

Many aspects of the real-time PCR procedures described here are instrument and protocol-specific, such that analytic sensitivity, specificity, primer selection and all aspects of the operating program would have to be validated separately for each method and instrument. The LightCycler 2.0 is used for real-time PCR for all of the pathogenic *Mycoplasma* and *Ureaplasma* species. Detailed descriptions for performing the assay to detect and differentiate the two *Ureaplasma* species in clinical specimens are provided in Table 16.6.

16.5.4 Quality Control

Careful attention to quality control procedures should limit the risk of false-positive and false-negative results of PCR assays. False-positive results from contamination are a major problem for conventional PCR but have been minimized with real-time PCR. The carryover contamination can be eliminated by addition of uracil-DNA glycosylase (UNG) to the master mix. In addition to human errors, reasons for

false-negative results include the presence of PCR inhibitors in the clinical specimen, suboptimal reagent preparation and reaction conditions, and inefficient extraction of the target DNA. Many substances present in clinical samples, such as hemoglobin, polysaccharides in sputum and mucolytic agents, certain compounds used for DNA extraction, such as ethanol or detergents, are potent amplification inhibitors and even swabs such as calcium alginate and those with aluminum shafts [46]. These inhibitory factors and suboptimal PCR conditions can be detected by simply mixing a positive control DNA with the sample after purification. However, this external control strategy cannot reveal inefficient DNA extraction. In this case, use of an internal control overcomes the limitations of the external control. The internal control can be a plasmid or oligonucleotide containing a sequence similar to or unrelated to the target, but can be differentiated from the assay PCR. The internal control is added directly to the crude sample and co-processed for purification and amplification along with the sample. This type of internal control is the most accurate method to modulate the important steps of diagnostic PCR protocols. It is essential in the initial validation of a PCR assay that all aspects that can potentially impact the results must be optimized so that once the assay has been validated the quality control procedures for individual runs will not be overwhelming.

16.5.5 Determination of Assay Sensitivity and Specificity

The PCR analytical sensitivity test should be performed against serial dilutions of template DNA, either bacterial genomic DNA from a defined inoculum titer or a plasmid containing the target sequence and can be expressed in terms of amount of DNA detected or numbers of organisms (CFU). The analytical specificity should also be tested against other pathogens that appear in the same body locations or show sequence similarities to the targets. Human genomic DNA should always be included in the evaluation because of its presence in clinical specimens and possible inhibitory effects. When appropriate primers and used and optimum PCR conditions are established, assays should be capable of detecting just a few organisms in a clinical specimen. For example, real-time PCR assays for *Ureaplasma* spp. detection and differentiation may have detection limits of 5–10 copies/reaction mixture [29, 156, 158, 181]. The assay reproducibility should be verified by testing the same samples multiple times.

The choice of which genes to use as PCR or other NAAT targets for optimum detection and characterization of the desired organism or component should be carefully considered. Too often the PCR targets used in early *Mycoplasma* and *Ureaplasma* assays were directed towards genes encoding antigens that upon additional study have proven to have wide sequence variation among different isolates. Consider the genes encoding P1 in *M. pneumoniae*, MgPa in *M. genitalium*, and MBA in ureaplasmas. In hindsight, all of these are poor PCR targets that are likely to yield erroneous results as a result of sequence heterogeneity and phase variation. Comparison of the G+C% of essential and nonessential genes in *M. genitalium*

showed that genes with lower G+C% were more likely to be nonessential [182]. More recently, the same trend was observed for *M. pneumoniae, M. alligatoris*, *M. mycoides* subspecies *Capri*, and *M. capricolum* subspecies *capricolum* (Glass, unpublished results). Critical genes for the function of these species tended to have the highest G+C%. All of these mycoplasmas, perhaps with the exception of *M. pneumoniae*, are under pressure to convert GC base pairs to AT base pairs. This is especially true at the third base in codons. Thus, if PCR targets are chosen at least in part based on having high G+C%, they may be more likely to have sequences conserved among all isolates for a given species of *Mycoplasma* or *Ureaplasma*.

Finally, it is important to use the assay on well characterized clinical specimens in either a prospective or retrospective manner that are also tested by other acceptable methods such as culture and/or serology to determine how well it performs. Before a laboratory can begin routine PCR-based diagnostic work, it must be able to demonstrate that its molecular results compare favorably or exceed detection ability of conventional culture-based techniques to establish a clinical sensitivity for the assay.

16.6 Discussion and Summary

The development and application of molecular-based methods for mycoplasmas and ureaplasmas in human infections over the past two decades has significantly improved the ability to detect, identify and genetically characterize these organisms. This has resulted in expansion of knowledge about the diseases they may cause and enabled more rapid and accurate diagnosis for clinical diagnostic purposes. This has been especially true for *M. pneumoniae* and *M. genitalium*. NAAT-based detection methods have lessened the reliance on the problematic serological detection systems that were previously the only feasible means for identifying persons with acute *M. pneumoniae* infection. The enthusiasm for development of NAAT-based systems for application in mycoplasmology has resulted in dozens of published assays using a broad array of gene targets and methodologies that have now become preferable to older nonmolecular or non-amplified methods in most instances. When used for diagnostic or epidemiological purposes in a clinical setting, there is justifiable concern over accuracy since most assays have never been sufficiently validated against other molecular or culture-based methods to ensure their accuracy. Since none of these assays has thus far been evaluated and approved by the US Food and Drug Administration, though several are sold commercially in some countries, much is still unknown about their sensitivity and specificity. The few comparative clinical studies of various NAATs and preliminary studies of interlaboratory proficiency testing have indicated there are considerable differences with these assays for detection of mycoplasmal infections, as well as the capabilities of the individual testing laboratories. It is clear that the future of diagnostic mycoplasmology and epidemiological research rests with molecular-based technology, even though culture, phenotypic methods, and traditional antimicrobial susceptibility testing will still have an important role, especially for *M. hominis* and *Ureaplasma* spp. Therefore,

it is very important that large-scale comparisons must be performed to compare reproducibility and accuracy of NAATs. This must include side-by-side comparisons of new assay formats and gene targets with existing assays using the same as well as different targets and with other established methods including culture when feasible. Such comparisons should ideally include a broad selection of specimen sources from different geographic areas. Eventually it seems likely that commercial development of NAATs for *M. pneumoniae, M. genitalium*, and perhaps *M. hominis* and *Ureaplasma* spp. will come to the United States as it has to Europe. Standardization of reagents and rigorous quality control would then be more realistic.

As noted earlier in this chapter, Next-Generation DNA sequencing from an increasing variety of technologies makes it possible to sequence microbial genomes inexpensively and accurately. Genome sequencing that took years a decade ago can now be done in a few hours for only a few percent of the investment once required. Thus, it is now possible to sequence and compare the genomes of scores of different microbes isolated from patients. This time and financial investment will no doubt continue to fall. As a result, we will soon be able to sequence the genomes of all interesting patient isolates. Already a single bacterial cell contains the entire template DNA required to obtain a genome sequence. The other sequencing change on the near horizon is cheap quick human genome sequencing. In concert, the advent of inexpensive bacterial and human genome sequencing will enable a new era in diagnosis. Some combinations of human genotype and pathogen genotype will demand one level of medical intervention. Other combinations may require no action other than surveillance. Methods for the detection of pathogenic mycoplasmas will continue to advance. Just as we have largely abandoned serological and culture-based detection of some of these atypical bacterial pathogens, no doubt some of the methods described here will likely become relics in the face of improved technology.

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Chapter 17
Corynebacterium diphtheriae

Igor Mokrousov

17.1 Introduction

Diphtheria is a rare disease caused by toxigenic strains of *Corynebacterium diphtheriae* and, less often, *Corynebacterium ulcerans*. The *Corynebacterium* species is a rod-shaped bacterium having a high GC content and classified into *Actinomycetales*, an order of Gram-positive bacteria, containing *Mycobacterium tuberculosis* and *Streptomyces coelicolor*. *C. diphtheriae* species comprises four biotypes, var. *gravis*, var. *mitis*, var. *intermedius*, and var. *belfanti*. All biotypes, with the exception of the biotype *belfanti*, may produce the lethal diphtheria exotoxin. After infection, *C. diphtheriae* can colonize the skin and/or the upper respiratory tract where it releases the toxin, causing the symptoms of the disease (reviewed in ref. [1]).

Substantial endemic circulation of toxigenic *C. diphtheriae* is observed in Africa, the Eastern Mediterranean, South America, Southeast Asia, and the Indian subcontinent as manifested by outbreaks or large numbers of reported cases [1, 2]. Importation of the microorganism from these endemic regions poses a constant threat, particularly among subgroups of individuals with low vaccination levels. At the same time, persistent foci of diphtheria exist in developed countries eventually causing small outbreaks [3, 4]. The reemergence of diphtheria in the newly independent states (NIS) of the former Soviet Union demonstrated the continued threat of this thought to be rare disease. Although following mass immunization campaigns and additional control measures, this epidemic is under control, the currently observed reemergence of toxigenic genotypic variants and circulation of invasive nontoxigenic strains appear alarming. These reasons highlight the importance of understanding of population structure of this pathogen and development of rapid, reproducible, and discriminatory typing techniques for epidemiological

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surveillance and global monitoring of *C. diphtheriae*. The prospective genotyping by classical DNA fingerprinting methods (MLEE [multilocus enzyme electrophoresis], PFGE [pulsed-field gel electrophoresis], and ribotyping) of the circulating isolates permitted to monitor the evolution of the Russian epidemic clone in the 1990s. However, some of these methods are time-consuming and rather cumbersome (MLEE, PFGE, and ribotyping), while others lack interlaboratory reproducibility and hence exchangeability of results (RAPD [randomly amplified polymorphic DNA]). To rapidly identify and monitor subtle changes in the genome structure at an intraclonal level during and between epidemics, fast, simple, portable, and discriminatory molecular typing methods of *C. diphtheriae* are still needed. In 2003, the first complete genome sequence of *C. diphtheriae* was published [5] that became a milestone achievement in genome research of this important human pathogen and offered new possibilities in search for new polymorphic markers for *C. diphtheriae* strain typing.

17.2 Methods

In the past, epidemiologic surveillance of diphtheria was limited by traditional typing systems, such as serotyping, phage typing, and bacteriocin typing ([2] and references therein) with low discriminatory power and insufficient reproducibility. In the 1990s, faced to the NIS/Russian epidemics, several laboratories in Europe and the USA successfully applied new molecular techniques, already developed for other bacterial species, to epidemiologic studies of diphtheria strains, especially those circulating in the NIS and neighboring countries.

17.2.1 Ribotyping

Ribotyping had previously been shown to be an extremely useful tool for DNA profiling of many bacterial species [6] and has been recognized as a straightforward method for typing *C. diphtheriae* isolates [2, 7]. The basic principle of this method is Southern transfer and hybridization of the digested chromosomal DNA with specific rRNA genes derived probes. In many bacteria rRNA operons are present in several copies in genome and the resulting multi-band patterns are frequently strain-specific. The number of fragments generated by ribotyping is a reflection of the multiplicity of rRNA operons present in a bacterial species. Initially, the entire *rrn* operons, e.g., those of *Escherichia coli* or *Bacillus subtilis*, were cloned into plasmid and used as hybridization probes. At present, a mixture of five oligonucleotides representing phylogenetically conserved regions in 16S and 23S rRNA genes (OligoMix5) is used for this purpose making the method a really universal approach for (eu)bacterial strain typing [8]; example of *C. diphtheriae* riboprofiles is shown in Fig. 17.1a. At the same time, recent in silico analyses showed that resolved DNA

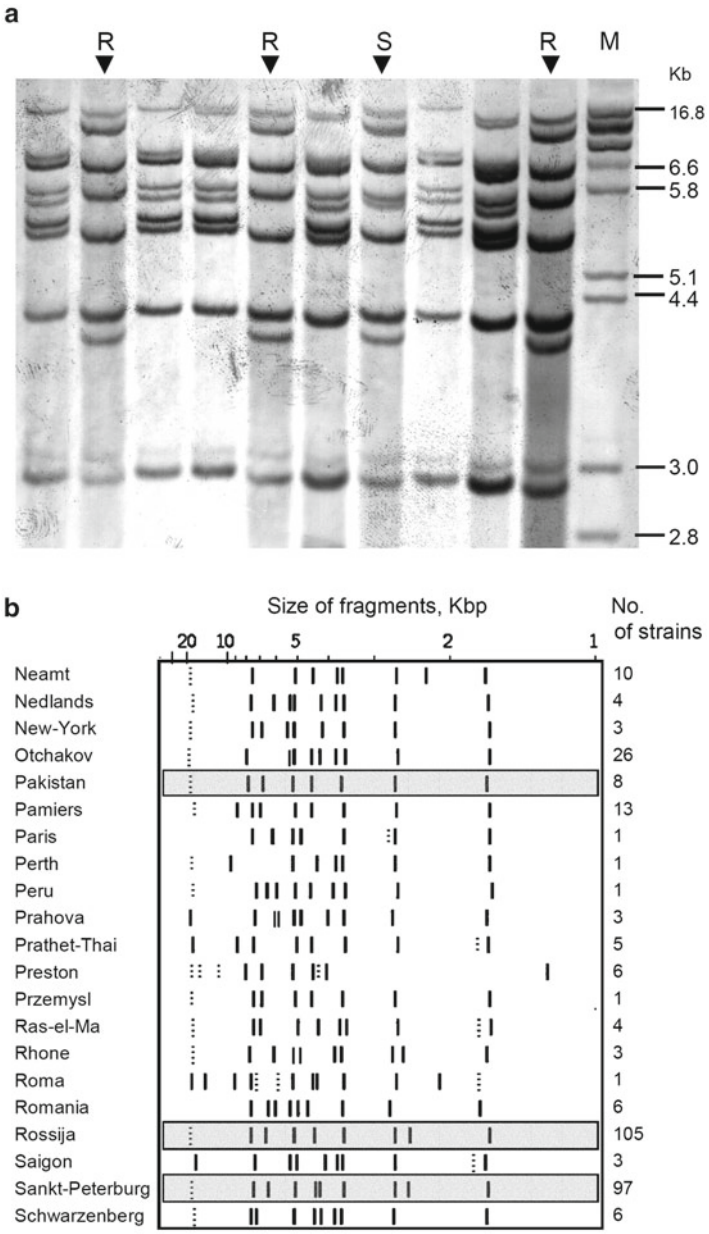

Fig. 17.1 *Corynebacterium diphtheriae* ribotyping. (**a**) *Bst*EII riboprofiles of some *C. diphtheriae* strains. *Arrows* indicate ribotypes Sankt-Peterburg (S) and Rossija (R) of the Russian epidemic clonal group. M: molecular weights marker, *Citrobacter koseri* CIP105177 DNA cleaved with *Mlu*I. (**b**) Extract of the international ribotype database in Institut Pasteur, Paris [10]: a schematic view obtained after computer processing of the profiles. Ribotypes Rossija, Sankt-Peterburg, and likely related Pakistan are gray-shaded. Reprinted from ref. [23] by permission of Elsevier ©2009

polymorphisms rather reflect restriction fragment length polymorphisms (RFLPs) of the neutrally evolving housekeeping genes typically found to flank chromosomal rRNA gene sequences [9]; this, however, does not reduce but rather highlights the phylogenetic value of ribotyping.

Detailed technical description of the method is given by Regnault et al. [8]. Some practical issues must be mentioned. First, the hybridization buffer may be either in-house traditional solution (SSC, SDS, sarkosyl, and blocking reagent [8]) or commercially available ready to use DIG EasyHyb (Roche). The latter permits using reduced temperatures for hybridization (42°C); however, it is rather sensitive to storing conditions and may generate strong background when expired. Second, five oligonucleotides included in the OligoMix5 were initially labeled at 3′-end with digoxigenin (DIG) using labeling kit. At present, it seems more practical and reliable to use commercially DIG-labeled oligonucleotides. Regarding the probe itself, OligoMix5 is a recommended one although previously used complete rDNA operon probe was shown to produce comparable results [8]. Third, a choice of restriction enzyme is a critical issue for development of the discriminatory and reliable scheme. *Pvu*II and *Bst*EII were shown to generate similar discrimination of *C. diphtheriae* strains, but *Bst*EII was ultimately retained since it generated much better interpretable patterns presenting a wide range of fragment sizes.

The hybridization profiles may be visualized as banding patterns on a membrane with an alkaline phosphatase (Roche Applied Science)-catalyzed colorimetric reaction (Fig. 17.1a); *Citrobacter koseri* CIP105177 DNA cleaved with *Mlu*I is used as molecular weights marker. Further, the membranes are scanned and profiles can be processed with TAXOTRON (Institute Pasteur, Paris) or Bionumerics (Applied Maths, Belgium) packages. One should always keep in mind a problem of comparison of banding profiles obtained in different gel runs and laboratories, but this is inherent to all analyzes dealing with banding profiles.

Efficient surveillance of the circulating *C. diphtheriae* variants would not be possible without international ribotype database that makes a good example of the long-term concerted efforts of many collaborating laboratories [10] within the frames of the WHO supported European Laboratory Working Group on Diphtheria (ELWGD) and the European Commission DIPNET project (http://www.dipnet.org). The nomenclature of *C. diphtheriae* ribotypes was published in 2004: a total of 86 ribotypes obtained after *Bst*EII digestion and hybiridization to OligoMix5 were given a geographic name chosen to reflect the place where one of the strains was isolated or studied [10] (Fig. 17.1b). Two patterns are considered identical when they are composed of the same number of fragments and, for homologous fragments, size differences are below a 5% threshold error value. In each reference pattern, each fragment size is calculated as the average of sizes of homologous fragments observed in the corresponding cluster. This numeric approach is not intended for phylogenetic inferences or taxonomic definitions but only to identify identical or similar patterns. The ELWGD principles for naming *C. diphtheriae* ribotypes were agreed as follows: (1) a ribotype name

should be unique; (2) each ribotype name is associated with a reference strain made available from a culture collection affiliated with the World Federation of Culture Collections; (3) once validated, a *C. diphtheriae* ribotype is given a geographic name taken from the place where the strain was isolated; (4) names are labels only and do not imply that a ribotype originated in the area used for naming it [10].

A traditional, manual ribotyping performed in many laboratories is labor-intensive and time-consuming. To solve these problems, an automated ribotyping has been established using RiboPrinter (Dupont Qualicon) automated ribotyping system that became a technological breakthrough with respect to convenience, reproducibility, and speed. At the same time, the higher speed inherently results in the relatively shorter-run of agarose gel format thus reducing quality of bands separation and discrimination. In this sense, traditional larger (16-cm length) gels generate much better resolution. In addition, the cost of ribotyping is significantly greater using the automated system.

17.2.2 Pulsed-Field Gel Electrophoresis

In this classical DNA fingerprinting method the genomic DNA is prepared to remain intact using lysis (lysozyme, RNase, and proteinase K) of bacterial cells directly in agarose blocks, followed by deproteination in proteolysis buffer. For *C. diphtheriae* typing, the DNA is cleaved with rare-cutting enzyme *Sfi*I and PFGE is carried out in TBE 1.5% agarose gels at 14°C by using a CHEF DRII system (Bio-Rad) with pulse times 5–20 s over 20 h and 1–5 s over 18 h. A lambda DNA concatemer (Bio-Rad) is used as a molecular size marker. The bands are visualized by staining the gel in ethidium bromide. Difference of three or more bands is used to distinguish PFGE types. *C. diphtheriae* PFGE typing can be performed according to the procedure described by De Zoysa et al. [7].

In principle, PFGE is highly reproducible and produces well-resolved fragments representing the entire bacterial chromosome in a single gel. It has been assumed to be the most discriminating of the currently available genotypic methods. However, in reality, PFGE was reported to be less discriminating than ribotyping when applied to isolates of *C. diphtheriae*: PFGE was not able to distinguish between the two main ribotype patterns in Russia [7, 11]. Furthermore, unlike conventional ribotyping, the molecular genetic basis for a detected PFGE profile is inherently imprecise for a number of reasons: (1) restriction endonuclease sites are unpredictably scattered throughout the chromosome and (2) unlike ribotyping, detected polymorphisms may involve any functional category of nonneutral genes, including those under the pressure of diversifying antigenic selection [9]. Although PFGE results can address the question "identical or not?," indexing the degree of identity between any two isolates with variant PFGE profiles remains uncertain.

17.2.3 Multilocus Enzyme Electrophoresis

The other important method used for *C. diphtheriae* strain typing is MLEE. This method detects amino acid substitutions affecting charge and conformation in cellular housekeeping enzymes. Such mobility variants, or electromorphs, of the same enzyme can be visualized by their activity in a starch gel matrix as bands of different migration rates. Each electromorph is considered to represent a distinct allele of that enzyme. A profile of electromorphs that define the electrophoretic type (ET) of each strain is obtained by testing 27 different enzymes. Finally, on the basis of these data, the genetic distance between the strains is calculated and presented as a dendrogram [12]. Although MLEE is a powerful and valid technique, it is not ideal, since comparisons of the results obtained in different laboratories are problematic, and the relationship between nucleotide sequence variation and isoenzyme variation typically is unknown.

17.2.4 PCR-Based Genotyping Methods for Rapid Screening

17.2.4.1 RAPD Typing

PCR with random (also named arbitrary or universal) primers has become a very popular approach for typing many pro- and eukaryotic species since 1990. In this polymerase chain reaction-based method, palindromic DNA structures are amplified from different genome regions using a single nonspecific primer. Different primers target different genome regions and may disclose different degree of interstrain genetic variation (see examples of RAPD profiles in Fig. 17.2). The use of crude DNA preparations of *C. diphtheriae* cultures was shown to result in poor amplification and the RAPD patterns were not reproducible. Furthermore, different thermal cycler models produced different RAPD patterns from the same DNA sample. However the reproducibility of the technique was good when the same thermal cycler was used throughout [13]. When applied to the Russian epidemic clonal group, RAPD had the lowest discriminatory power compared to MLEE and ribotyping [14]. At the same time, this discriminatory "inability" implied a possibility to use RAPD typing for preliminary screening of *C. diphtheriae* isolates and rapid and unambiguous identification of the epidemic clone [14–17].

17.2.4.2 AFLP Typing

Another method also widely used for typing bacterial pathogens and subsequently applied to *C. diphtheriae* is amplified fragment length polymorphism (AFLP) analysis that is based on the selective PCR amplification of genomic restriction fragments

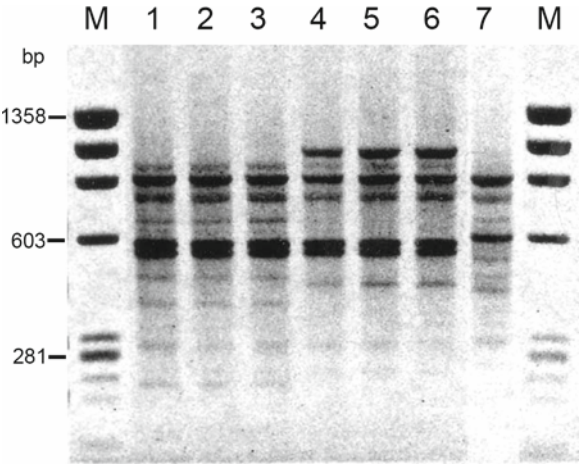

Fig. 17.2 Example of RAPD profiles of *Corynebacterium diphtheriae* strains from St. Petersburg, Russia. M: molecular weight marker phiX174/*Hae*III

of the whole genome [18]. Classically, the technique involved three steps: (1) restriction of the DNA and ligation of oligonucleotide adapters, (2) selective amplification of sets of restriction fragments, and (3) gel analysis of the amplified fragments. PCR amplification of restriction fragments is achieved by using the adapter and restriction site sequence as target sites for primer annealing. The selective amplification is achieved by the use of primers that extend into the restriction fragments, amplifying only those fragments in which the primer extensions match the nucleotides flanking the restriction sites. Using this method, sets of restriction fragments may be visualized by PCR without knowledge of nucleotide sequence. The method allows the specific co-amplification of high numbers of restriction fragments. The number of fragments that can be analyzed simultaneously, however, is dependent on the resolution of the detection system. Typically 50–100 restriction fragments are amplified and detected on denaturing polyacrylamide gels.

De Zoysa and Efstratiou [19] used a simplified version of that technique, utilizing a one-step digestion-ligation reaction with one enzyme, and the PCR with a single primer; consequently, a relatively small number of amplified bands could be separated by agarose gel electrophoresis. Strains with ribotypes Sankt-Peterburg and Rossija (epidemic clone) and ribotype Pakistan could not be distinguished; however, the technique discriminated isolates of ribotypes Vladimir, Lyon, and Otchakov. The reproducibility of the method was examined by using two different thermal cyclers and duplicate AFLP runs for each isolate with two separate DNA extractions. Under all these different conditions, the fragments for each AFLP profile were identical, although variations in the intensities of some of the bands were observed with different PCR runs. The technique analyzes the whole genome, requires only a small amount of DNA, and requires no prior sequence information about the target DNA.

AFLP was suggested to have the potential to replace ribotyping as the "gold standard" within the ELWGD [19].

17.2.4.3 New Generation Molecular Markers

Publication of the first complete genome sequence of *C. diphtheriae* strain NCTC13129 [5], along with use of high-throughput 454/Solexa technologies for partial genome sequencing [20] have greatly accelerated the development of new typing approaches for *C. diphtheriae*.

17.2.4.4 Multilocus Sequence Typing

Multilocus sequence typing (MLST) is proposed as a procedure for characterizing isolates of bacterial species using the sequences of internal fragments of seven housekeeping genes. MLST is based on the well-established principles of MLEE, but differs in that it assigns alleles at multiple housekeeping loci directly by DNA sequencing, rather than indirectly via the electrophoretic mobility of their gene products (http://www.mlst.net). Several criteria are used in the selection of all potential loci. Genes included are those encoding putative housekeeping products necessary for biological roles in DNA repair, replication, and amino acid biosynthesis. Genes that are either located near or implicated as being putative virulence factors and mobile elements should be avoided, since these may come under greater selective evolutionary pressures than other genes. The selected loci should be distributed as much as possible across the chromosome to ensure that each locus was genetically unlinked. For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST). Each isolate of a species is therefore characterized by a series of seven integers which correspond to the alleles at the seven housekeeping loci.

The number of genes used for MLST is seven but they vary for different species, e.g., *adk*, *atpG*, *frdB*, *fucK*, *mdh*, *pgi*, *recA* are used for *Haemophilus influenzae*, *atpD*, *gltB*, *gyrB*, *recA*, *lepA*, *trpB,* and *phaC* for *Burkholderia cepacia* complex (http://www.mlst.net). Bolt et al. [21] recently reported MLST scheme (genes *atpA*, *dnaE*, *dnaK*, *fusA*, *leuA*, *odhA*, *rpoB*) to characterize global populations of *C. diphtheriae*, *C. ulcerans* and *Corynebacterium pseudotuberculosis*. In particular, 149 isolates of *C. diphtheriae* from 18 countries isolated during 50 years were analyzed by MLST and strain discrimination was generally in accordance with ribotyping data and clonal complexes associated with disease outbreaks were identified. Moreover MLST showed divisions between *gravis*/*mitis* and *belfanti* and evidence of novel veterinary subgroups.

17.2.4.5 MLVA Typing

Multi-locus VNTR (variable number of tandem repeats) analysis MLVA is based on variation in copy number in the polymorphic VNTR loci scattered throughout the bacterial chromosome; this approach is widely used for various bacterial pathogens including relatively close relative of *C. diphtheriae*, *M. tuberculosis*. The number of repeat copies per locus may vary among strains, and the use of several such loci allows sufficient interstrain differentiation. The VNTR profiles are presented as multi-digit numerical codes ("complex haplotypes"), each digit representing the copy number in a locus. In fact, the VNTR loci present multiple independent genetic markers and therefore ideally suit for phylogeographic analysis. At present, MLVA typing of *C. diphtheriae* is still under development [22, 23, 23a].

17.2.4.6 CRISPR-(spoligo)typing

CRISPR (clustered regularly interspaced short palindromic repeats) regions are found in many bacteria and consist of 20–40 bp direct repeats (DR), interspaced by similarly sized non-repetitive variable spacers (Fig. 17.3a). In the complete genome sequence of *C. diphtheriae* strain NCTC13129, two CRISPR loci are located 39 kb downstream and 180 kb upstream of the *oriC* region [24]. The first locus (DRA) consists of seven units; the second DR locus (DRB) consists of 27 DRs and 26 spacers whereas some of them are duplicated.

The recently developed method to study polymorphism in the DRB and DRA CRISPR loci is based on a reverse hybridization in macroarray format [24, 25] (Fig. 17.3b). Analogously to the spoligotyping method used for *M. tuberculosis* analysis, we suggested using the same name for this assay for *C. diphtheriae* subtyping. The specific oligonucleotides (5′-amino labeled) were designed on the basis of the 21 and 6 different spacers sequences found in the DRB and DRA regions in *C. diphtheriae* strain NCTC13129. The probes were chosen to have similar melting temperatures and are covalently bound to a membrane. All spacers of each DR locus are amplified with a locus-specific single primer pair, the reverse primer being 5-biotin labeled (Fig. 17.3a). The biotin-labeled PCR fragments of the both CRISPR regions are co-hybridized to the set of the 27 spacer-derived probes (21 DRB and 6 DRA spacers) by using the MN45 miniblotter providing a macroarray format. The autoradiographs (hybridization profiles on the chemiluminescence-sensitive ECL film [Amersham Biosciences, UK]) are visually assessed for presence/absence of signals (Fig. 17.3b). The obtained hybridization profiles of 21 (DRB locus) and six (DRA locus) signals are entered into spreadsheet in binary format; this allows further simple comparison of profiles by automatic sorting function.

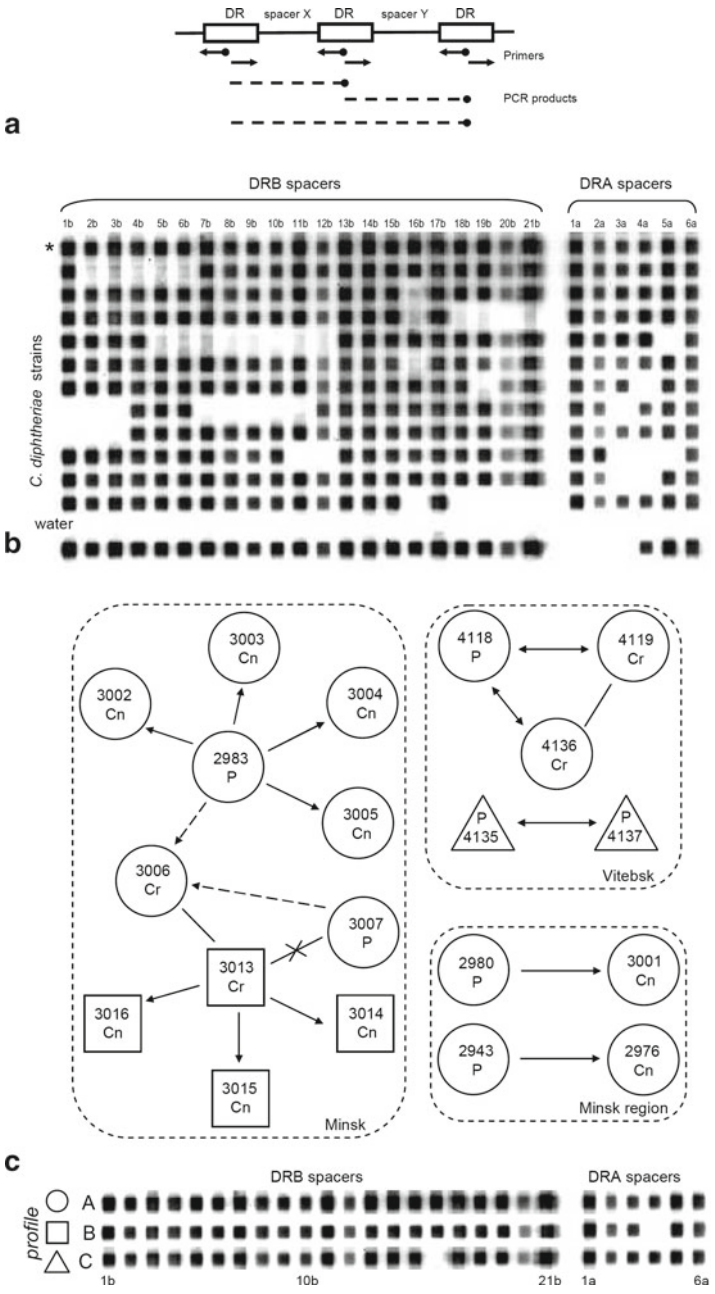

Fig. 17.3 *Corynebacterium diphtheriae* spoligotyping. (**a**) PCR of a CRISPR locus with a locus-specific DR-sequence-defined one primer pair that amplifies all present variable spacers. Reverse primer is labeled with biotin (shown as *black dot*); this permits detection of hybridization signals via streptavidine-peroxidase mediated chemiluminescence on a light-sensitive film. (**b**) Examples of spoligoprofiles of the Russian strains of the epidemic clone; *asterisk* designates the ancestral profile T1 with all 27 signals present. Reprinted from ref. [23] by permission of Elsevier ©2009. (**c**) Combined conventional and molecular epidemiological investigation of the *C. diphtheriae* ribotype Rossija foci in Belarus. In the epidemiological linkage network individuals are linked based on standard investigation; these links were further rejected (*cross*) or confirmed (*solid line*) by spoligotyping analysis of strains. P, patient, Cr, carrier, Cn, contact. Reprinted from ref. [28] by permission of Springer ©2009

17.3 Applications

17.3.1 Origin and Dynamics of the Epidemic Clone in the 1990s

In the 1990s molecular typing methods allowed identification of a clonal group of closely related strains responsible for the epidemic in Russia and NIS countries and to trace strains imported to other countries [2, 7, 14]. These strains were indistinguishable by PFGE, RAPD, AFLP and very similar in ribotyping two principal profiles, Rossija and Sankt-Peterburg, differing in one band (Fig. 17.2) [2, 7, 10, 19]. Minor rare variants were identified by RAPD and ribotyping techniques [14] and a total of 27 ET types similar at >80% were described by MLEE typing in all strains of this clonal group [2]. During diphtheria epidemic in 1990–1996 these closely related toxigenic strains were isolated in a high proportion (70–90%) of patients in all NIS countries and Russia; few strains were also identified in other European countries as imported cases [2, 7, 14, 17]. Prospective and retrospective studies using MLEE, ribotyping and PFGE showed that (1) the pre-epidemic period was characterized by the simultaneous presence of many different ETs; (2) the epidemic clonal group had a unique PFGE profile and comprised MLEE-defined ET8 complex strains of the ribotypes Sankt-Peterburg and Rossija [2]. These ribotypes were less frequently seen in Russia before the epidemic. However, since 1991, they have accounted for an increasing proportion of the isolates studied and by 1994, they accounted for 80% of all identified ribotypes.

The population of *C. diphtheriae* was not constant throughout the epidemic process. As the epidemic became widespread, new geographic variants within the ET8 complex emerged, while more evolutionarily stable ribotypes remained unchanged [2]. In Russia, a steady decline of diphtheria incidence since 1997 was accompanied by increasing heterogeneity of the *C. diphtheriae* population. Although different ribotypes were identified in Russian archival strains recovered in 1940–1990, only one ribotype was prevailing in each particular period: Lyon in 1940–1960, Otchakov in 1980s, Sankt Peterburg and Rossija since mid-1980s until peak in mid-1990s (96% in 1996). Since 1997, during the period of lower incidence, Sankt Peterburg and Rossija became less common (77% in 2001), and other ribotypes became more prominent (Otchakov, Lyon, Cluj, Buzau) [26].

In Belarus, another NIS country affected by the diphtheria epidemic, mass immunization caused visible changes in the circulating population of *C. diphtheriae* [27]. The *gravis* biotype which prevailed in 1996–2000 was replaced with the *mitis* biotype in 2001–2005. Simultaneously, the proportion of toxigenic *C. diphtheriae* strains decreased from 47.1% (1996) to 6.8% (2005). Ribotyping analysis revealed the elimination of rare ribotypes (both toxigenic and nontoxigenic) during the period of decreased morbidity. In 2001–2005 not only "toxigenic" rare ribotypes were eliminated but also the proportion of "toxigenic" prevalent ribotypes decreased from 55.6% to 27.0%. However, the strains of these ribotypes that continued to circulate remained toxigenic. The proportion of Sankt-Peterburg ribotype in the total population decreased from 24.3% to 2.3%, in contrast, the proportion of the Rossija ribotype increased from 25.1% to 49.1% [27].

In the mid-1990s, imported cases of diphtheria contracted in Russia and Ukraine by foreign travelers were diagnosed in Finland, Estonia, Norway, Poland, Latvia, Lithuania, and Germany ([7], and references therein). However, such importation of toxigenic clones not always occurred—even through the borders of the neighboring countries—thus underlying a critical importance of the vaccination coverage and host susceptibility. For example, Romania has a sufficiently long border with the former Soviet Union countries (Ukraine and Moldova), hence opportunities for strain importation. However, the Russian/NIS epidemic did not spread to Romania owing to enforced prevention and control measures [17].

17.3.2 Global Diversity of C. diphtheriae

More distant world regions, such as UK, USA, and Canada were less influenced by Russian epidemics, but instead, via epidemiological links with other world areas reflecting long-term historical links. From 1986 to 1997 in the UK, there were 45 cases of toxigenic *C. diphtheriae* infections that were imported from Africa, Asia, Europe, and Eastern Europe. Most imported cases were caused by the biotype *mitis* and comprised many ribotypes not seen within the Eastern European region. Those ribotypes appeared to be unique to those particular countries, e.g., the ribotypes from cases imported from Thailand were identical to those of the epidemic isolates in Thailand and Laos. To compare the isolates collected during the recent Russian diphtheria epidemic with those circulating worldwide, isolates from Russian and previous epidemic areas (Thailand, Vietnam, Sweden, and the USA) were examined by ribotyping along with sporadic isolates from cases and contacts in the West Indies, France, Italy, Denmark, Romania, Rwanda, and Australia. An assessment of the transcontinental spread of the organism showed that several genotypes of *C. diphtheriae* circulated on different continents of the world and that each outbreak was caused by a distinct clone. The ribotypes seen in Europe appeared to be distinct from those seen elsewhere, and certain ribotypes appeared to be unique to particular countries [7, 11].

Another study used higher-resolution MLEE typing to determine the genetic relatedness of *C. diphtheriae* strains from Russia and NIS and worldwide (Australia, Bangladesh, Ecuador, Finland, Somalia, Sweden, Tunisia, and the USA) [2]. In addition to the ET8 complex (approximate epidemic isolates from Russia and NIS), two more clusters were observed. The first cluster (0.22 dissimilarity), comprised isolates from diphtheria patients and carriers in several US states from 1973 to 1996. These *C. diphtheriae* isolates were clearly distinct from the current epidemic isolates from Russia and NIS. Clustering of older and recent US isolates suggested an endemic focus of toxigenic *C. diphtheriae* in the USA. The other cluster (0.14 dissimilarity) included isolates collected in 1992 in Australia. The rest of the isolates were spread throughout the dendrogram without particular association between ET and geographic or temporal origin of strains [2].

At the same time, persistent foci of diphtheria do exist in developed countries eventually causing small outbreaks. Despite the virtual elimination of diphtheria in

the USA and Canada, toxigenic strains continue to circulate in some communities within the two countries. Molecular characterization of USA (South Dakota) and Canadian (Ontario) *C. diphtheriae* isolates showed that strains with characteristic molecular subtypes have persisted in these areas for at least 25 years [3, 4]. The enhanced surveillance in South Dakota revealed that toxigenic *C. diphtheriae* is circulating among American Indian populations [3].

17.3.3 Comparison of Methods

The traditional molecular methods used for various bacteria and applied to *C. diphtheriae* typing were ribotyping, PFGE, and MLEE [2, 7]. In spite of their wide use, these techniques are time-consuming, require specialized equipment and technical expertise, and, therefore, cannot be performed in all laboratories (Table 17.1). In contrast, the PCR-based methods are faster and simpler, although they frequently lack sufficient discriminatory power and reproducibility and their standardization is a challenge. De Zoysa et al. [11] compared four typing methods and found ribotyping to be highly discriminatory and reproducible. The statistical analysis data calculated for the different typing methods indicated that ribotyping is the most suitable technique and the method of choice for the typing of *C. diphtheriae*. The two PCR-based techniques, RAPD and AFLP, proved to be rapid and easier to perform than ribotyping and PFGE. The diversity indices indicated that AFLP and PFGE are less discriminatory than ribotyping and RAPD. RAPD and AFLP were suggested as rapid methods which can be used as screening techniques, prior to ribotyping, during outbreak investigations [11].

Unlike the above traditional typing methods, CRISPR-based spoligotyping of *C. diphtheriae* was evaluated in only two settings. A large number of variable characters (i.e., particular spacers that may be present or absent in a CRISPR locus) should provide sufficient level of variation to differentiate clinical strains. Indeed, a spoligotyping study of the Russian strains of the epidemic clone revealed an astonishingly high diversity. The 156 Russian strains of the epidemic clone from St. Petersburg, 1997–2002, were subdivided into 45 spoligotypes compared to only two ribotypes (Sankt-Peterburg and Rossija). The larger DRB locus (21 spacers) demonstrated higher polymorphism. Nevertheless, a combined use of the two loci additionally contributed to further strain differentiation (compared to use of the DRB locus alone) [25]. Interestingly, ribotype Sankt-Peterburg was found to be more heterogeneous than Rossija, in both CRISPR loci alone and in their combination [24, 25]. The polymorphism in both CRISPR loci and spoligotype distribution within two ribotypes of the *C. diphtheriae* epidemic clone led us to suggest: (1) a monophyletic origin of the epidemic clone, (2) presently clonal evolution of these CRISPR loci in *C. diphtheriae* genome, and (3) divergence between ribotypes Sankt-Peterburg and Rossija in northwestern Russia. Assuming that more diversity is generated due to longer evolutionary history, ribotype Sankt-Peterburg appears to be evolutionarily older and ancestral to ribotype Rossija. This latter may have originated

Table 17.1 Comparison of methods used for *C. diphtheriae* strain typing

Method	Target	Rationale/principle	Type of data	Advantages	Disadvantages	References or website
Ribotyping	16S and 23S rRNA genes and flanking regions	Total genomic digestion followed by Southern hybridization with rDNA probe	Restriction fragment length polymorphism: patterns based on size and no. of fragments that contain rRNA genes	Good discrimination, reproducible	Time consuming, limited portability	[8, 10] http://www.dipnet.org/ribo.public.php
PFGE	Whole genome	Total genomic digestion with rarely cutting endonuclease followed by electrophoresis characterized by frequent changes in orientation of electric field	Restriction fragment length polymorphisms of total genomic DNA	Reproducible	Less discriminatory than ribotyping; labor-intensive; limited portability	[7, 17]
MLEE	Various loci, usually housekeeping genes	Allelic differences in mobility revealed by starch gel electrophoresis	Electrophoretic type: composite of allele types	Reproducible, discriminatory	Labor-intensive; limited portability	[2, 12]
AFLP	Whole genome	Selective PCR amplification of genomic restriction fragments	Patterns in agarose gel based on size and no. of amplicons	Rapid, inexpensive, reproducible	Less discriminatory than ribotyping	[19]
RAPD	Whole genome	Differences in abundance and distribution of annealing sites revealed by polymerase chain reaction	Patterns in agarose gel based on size and no. of amplicons	Rapid, inexpensive	Insufficient reproducibility	[13–16]

Spoligotyping	Two CRISPR loci	Reverse hybridization macroarray-based detection of spacers present in CRISPR loci	Digital binary profile (0/1 for presence/absence of particular spacers)	Highest discrimination; rapid, portable, inexpensive	Reproducibility and stability to be checked; use with other ribotypes beyond epidemic clone yet to be established	[24, 25]
MLST	Seven selected housekeeping genes	Partial sequencing	Multi-digit allelic profile	Rapid, portable, reproducible	Discrimination similar to ribotyping	[21] http://www.mlst.net/misc/new_schemes.asp
MLVA	Multiple VNTR loci	Size detection of PCR fragments by agarose gel or capillary sequencer to detect number of repeat units in a locus	Multi-digit allelic profile	Portable, easy, reproducible	Discrimination and stability should be tested	[22, 23, 23a]

from one particular subpopulation (ancestral type T1 [Fig. 17.3b]) of the presumably already heterogeneous ribotype Sankt-Peterburg, followed by subsequent independent non-homoplasious evolution of the DRB and DRA loci in both ribotype sublineages.

In another study, 20 *C. diphtheriae* biotype *gravis* toxigenic isolates collected in Belarus from suspected foci of diphtheria infection (diphtheria cases, carriers, or contacts) were subjected to ribotyping and spoligotyping [28]. All strains had identical ribotype profile Rossija based on comparison with international ribotype database in Institut Pasteur of Paris. However, spoligotyping based on analysis of two CRISPR loci differentiated these strains into three spoligotypes (Fig. 17.3c). Comparison of the spoligotyping results with the epidemiological linkage network helped us to resolve suspected links in the chains of transmission.

17.4 Conclusions

Since 1993, application of the traditional molecular subtyping methods and continuous monitoring of the spread of the epidemic clones had a significant public health impact making it possible to distinguish between epidemic, endemic, and imported cases and allowing for implementation of timely and adequate preventive measures. To improve such continuous monitoring of the circulation of existing clones and to be able to rapidly detect the appearance of new and unusual clones, the *C. diphtheriae* ribotype database has been established in Institut Pasteur, Paris [10] and is also available at DIPNET web site (http://www.dipnet.org). More recently, publication of the *C. diphtheriae* complete genome sequence and partial genome sequencing permitted to select seven housekeeping genes for MLST [21] and to identify two CRISPR regions [24, 25] and multiple VNTR loci [22, 23] as possible candidates for development of new generation typing formats of *C. diphtheriae*.

General and/or potential advantages of the CRISPR- and VNTR-based methods are (1) digital presentation of data as discrete binary or multistate characters and portability; (2) high discriminatory power; (3) possibility to use cell lysates instead of purified DNA; and (4) high-throughput capacity. The novel CRISPR-(spoligo)typing [25] and MLST [21] methods may become a powerful tool for portable and high-resolution epidemiological monitoring and phylogenetic analysis of *C. diphtheriae.*

The critically important issue regarding these new methods is their field evaluation in both particular settings and in global collection. For example, it is not surprising that a collection of the geographically diverse strains was well differentiated by MLST [21]. On the other hand, although spoligotyping achieved excellent discrimination within the Russian epidemic clone [24, 25], the method should be validated in other settings. Furthermore, development of the CRISPR typing should include new spacers and other circulating variants of *C. diphtheriae* beyond the epidemic clone and investigate the spoligoprofile stability in short- and middle-term natural and laboratory-modeled evolution. Indeed, MLST, VNTR, and CRISPR

encoded information is easily databasable. Standardization of these new methods and building of a comprehensive database representing various markers and different levels of genetic diversity of *C. diphtheriae* and including microbiological/clinical strain/patient data is a task and challenge for near future. Availability of the very recently published new complete genomes of *C. diphtheriae* strains representing different biotypes [29–31] will provide new in-depth insights into pathogenomics and evolutionary history of this important human pathogen.

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Chapter 18
Burkholderia

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18.1 Introduction

Historically, the most well-known *Burkholderia* species were arguably *Burkholderia pseudomallei* and *Burkholderia mallei* that were long recognized as serious infectious agents causing meliodosis and glanders, respectively. Their harmful role in human medicine has been further aggravated by concerns about their potential use in biological warfare. Today, infections with *B. mallei* are extremely rare in humans and meliodiosis occurs as an endemic disease in South East Asia, with many cases in Thailand. However, other *Burkholderia* species have emerged on a global scale and during the past 30 years started to pose a serious health risk to susceptible individuals. One of the most problematic groups of *Burkholderia* opportunistic pathogens are collectively referred to as *Burkholderia cepacia* complex (Bcc).

Infections with the Bcc organisms are most frequently reported in association with cystic fibrosis (CF), a genetic disorder where lower airways become obstructed with a dehydrated thick and viscous mucus. This altered lung environment allows Bcc bacteria to establish a chronic infection that can ultimately result in rapid lung function decline and fatal septicemia. The invasive ability of Bcc bacteria and their ability to cause systemic infection in CF individuals is unique and does not occur with other CF pathogens such as *Pseudomonas aeruginosa*. Interestingly, individuals with chronic granulomatous disease also suffer from infections with Bcc bacteria; however, the incidence of this human disorder is very rare. Other patients affected by the Bcc are typically represented by the immunocompromised or those who have

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suffered some form of trauma. Many such patients contract Bcc infection as a result of hospital-associated outbreaks, with contaminated disinfectants or other pharmaceutical products often implicated as the source of infection.

This issue of person-to-person transmissibility of the Bcc organisms had a great impact on the standards of care for CF patients in terms of introducing strict segregation policy in hospitals. In order to identify Bcc outbreaks, to tackle spread of the infection within CF communities and to carry out surveillance, molecular typing systems became an essential analytical tool for epidemiologists and clinical microbiologists. In addition, the need to track infection was further complicated when isolates of bacteria initially identified as *B. cepacia* were found to comprise multiple novel species. As a result, novel typing schemes also contributed substantially to accurate identification of the Bcc species and to clarification of the taxonomy of the complex.

18.2 Taxonomy and Species Identification

The genus *Burkholderia* was only created in 1992 when several isolates of *Pseudomonas* including *Pseudomonas cepacia* were found to belong to a distinct rRNA gene homology group. The genus has expanded enormously over the last two decades, and today it contains 67 species. However, many of the constituent species include only environmental isolates which have little or no role in human medicine. In contrast, the medically important group of *Burkholderia* organisms is undoubtedly the *B. cepacia* complex which currently comprises 17 species (Table 18.1). They all are naturally present in the environment, but can also cause serious infections in vulnerable individuals.

The term "complex" underlines close phenotypic and genotypic relatedness between the species involved and indicates potential troubles with their correct identification. Phenotypic methods are insufficient in exact diagnostics of the infection as they fail to identify individual Bcc species; moreover, they can have substantial problems already with correct detection of Bcc *sensu lato*. It was the pioneering studies of Vandamme et al. in 1997 [1] that first recognized *B. cepacia* isolates comprised several species, or genomovars as described originally, and that multiple tests known as a polyphasic approach were needed to correctly define the taxonomy of the complex.

Definitive identification of each Bcc species is now heavily dependent on the use of molecular genetic methods. Various protocols were described in the past including species-specific PCRs, marker gene restriction fragment length polymorphism (RFLP) and gene sequence analysis; however, as the genetic heterogeneity within the Bcc has extended remarkably over the years, several molecular approaches have now been found to be suboptimal. Today, a PCR that targets the housekeeping gene *recA* is widely used and considered a gold standard for identification of the Bcc as a whole [2], although it is vital that the correct amplicon size of 1,044 bp is obtained in this test. Accurate identification of each species within the Bcc should be based on sequence analysis either of all seven genes that are part of multilocus sequence

Table 18.1 Taxonomy of *Burkholderia cepacia* complex

Species name	Former designation	Type strain
Burkholderia cepacia	Genomovar I	LMG 1222^T (ATCC 25416^T)
Burkholderia multivorans	Genomovar II	LMG 13010^T
Burkholderia cenocepacia	Genomovar III	LMG 16656^T (J2315^T)
Burkholderia stabilis	Genomovar IV	LMG 14294^T
Burkholderia vietnamiensis	Genomovar V	LMG 10929^T
Burkholderia dolosa	Genomovar VI	LMG 18943^T
Burkholderia ambifaria	Genomovar VII	LMG 19182^T (AMMD^T)
Burkholderia anthina	Genomovar VIII	LMG 20980^T (W92^T)
Burkholderia pyrrocinia	Genomovar IX	LMG 14191^T (ATCC 15958^T)
Burkholderia ubonensis	Genomovar X	LMG 20358^T
Burkholderia latens	BCC1	LMG 24064^T (FIRENZE 3^T)
Burkholderia diffusa	BCC2	LMG 24065^T (AU1075^T)
Burkholderia arboris	BCC3	LMG 24066^T (ES0263A^T)
Burkholderia seminalis	BCC7	LMG 24067^T (AU0475^T)
Burkholderia metallica	BCC8	LMG 24068^T (AU0553^T)
Burkholderia contaminans	Group K (BCC AT)	LMG 23361^T (J2956^T)
Burkholderia lata	Group K	LMG 22485^T (383^T)

typing (MLST) scheme (see Sect. 18.3.2 below) or, alternatively, of at least the *recA* gene. In light of these molecular genetic analyses it became apparent that the complex consists of species which share >98% sequence similarity in 16S rRNA gene and 94–95% sequence similarity in the *recA* gene. It was also noteworthy that none of the Bcc species presented a sequence divergence in its seven concatenated MLST genes that was greater than 3% [3].

18.3 Bcc Infection in CF: Why Genotype at the Strain Level

18.3.1 Early Epidemiology

The ability of Bcc bacteria to cause nosocomial outbreaks was recognized in the 1970s with isolates of "*P. cepacia*" implicated in various instances of disinfectant contamination; however, few of these instances of spread of infection were examined using molecular typing methods. It was the emergence of Bcc bacteria as devastating CF infections that forced the development of typing methods and created the need to genotype Bcc bacteria at the strain level. All of these early studies were performed prior to a detailed understanding of the taxonomy of this species; hence, we will refer to the undefined Bcc isolates in these studies as "*P. cepacia.*" The rapid emergence of "*P. cepacia*" as a CF pathogen was first recognized by researchers at a CF treatment center in Toronto, Canada, where between 1971 and 1981 the prevalence of infection rose from 10 to 18%, despite the level of *P. aeruginosa* infection remaining

Table 18.2 The pioneering application of strain genotyping to the epidemiology of Bcc bacteria in CF

Feature of CF infection/Bcc strain analysis	Typing or identification method applied	Useful reference or review
Epidemiology prior to designation of the Bcc		
CF center-specific Bcc strains	Ribotyping	[13]
Person-to-person transmission	Ribotyping	[5]
	PFGE	[6]
Intercontinental spread	MLEE/ribotying	[7]
Epidemiology with an understanding of the new Bcc taxonomy		
Identification of multiple species in the Bcc	Polyphasic taxonomy	[1]
Accurate molecular identification of Bcc species	*recA* sequence analysis	[2]
	MLST	[9]
National epidemiological surveys	*recA* sequence analysis	[14]
	RAPD	[15]
	PFGE	[16]
Clonality of environmental and clinical Bcc isolates	PFGE	[17]
	MLST	[18]
Global prevalence of a Bcc strain	MLST	[10]

constant among the same patient population [4]. It was also noted that a very high fatality rate of 62% was linked to these "*P. cepacia*" CF infections. Subsequently, several researchers noted that CF individuals were frequently infected with the same strain of "*P. cepacia,*" with many of these strains being specific to certain CF treatment centers and also able to spread from patient-to-patient via social contact outside of hospital.

One of the first methods to be applied in tracking the epidemiology of "*P. cepacia*" was ribotyping, where chromosomal RFLPs were detected by probing with the rRNA gene cluster. LiPuma and colleagues [5] used ribotyping to demonstrate the presence of CF center-specific strains and the ability of "*P. cepacia*" to spread between individuals with CF (Table 18.2). This study was the first to note that infection control measures such as cohorting or isolation of "*P. cepacia*" infected CF patients may be needed to limit the spread of infection during their hospitalization. Govan and co-workers [6] then added to this observation and used pulsed field gel electrophoresis (PFGE) to establish that social contact outside of hospital could also lead to transmission of "*P. cepacia*" in CF individuals (Table 18.2). These pioneering studies led CF microbiologists around the world to begin to genotype "*P. cepacia*" isolates and to define the molecular epidemiology of this pathogen in CF.

Multiple genotyping methods were developed and evaluated for application to "*P. cepacia*" CF isolates including ribotyping, PFGE, PCR-ribotyping, random amplified polymorphic DNA (RAPD) analysis and enterobacterial repetitive intergenic consensus (ERIC) PCR fingerprinting (often designated as a repetitive element PCR or rep-PCR fingerprinting method) (Table 18.2). In addition, several researchers also applied multilocus enzyme electrophoresis (MLEE) in combination with genetic typing methods to resolve the epidemiology of infection.

After the initial identification of CF center-specific isolates and Bcc transmission among CF individuals, the finding that one particular "*P. cepacia*" strain had also spread intercontinentally between North America and Europe presented a further worrying complication for the CF community. The strain had originally been identified by MLEE and given the designation as electrophoretic-type ET12 by Johnson et al. [7]. Subsequent analysis by ribotyping, PFGE and RAPD corroborated that this was a single strain type. The epidemiology that underlies the spread of the ET12 strain between continents has never been fully elucidated. The studies by Johnson et al. [7] suggested that contact between patients at Canadian CF summer camps may have led to the carriage of the ET12 strain back to the United Kingdom, and subsequent spread to mainland Europe. It is also highly likely that the rapid increase in prevalence of "*P. cepacia*" at a Toronto treatment center recognized by Isles et al. [4] 10 years prior was also due to the same ET12 strain. However, with no isolates being available for analysis from this early study, the epidemiology of ET12 cannot be fully elucidated beyond the work of Johnson et al. [7]. Taxonomic studies of ET12 isolates later revealed that they belonged to the species *Burkholderia cenocepacia* (Table 18.1) and with its virulent, highly transmissible properties this species has been widely studied in terms of molecular pathogenesis [8].

18.3.2 Epidemiology Post Genomovars

Results of comprehensive taxonomic studies led to more accurate selection of clinical isolates to be tested by molecular typing methods. Following a discovery of multiple genomovars (species) within Bcc, it became apparent that by far the most predominant species in CF patients were *B. cenocepacia* and *Burkholderia multivorans*; and these two became a main subject of interest for next epidemiological studies. Projects that mapped epidemiological situation either in a single CF center or on a national level were still using PFGE and/or RAPD methods, but also other genotyping schemes such as variants of rep-PCR (i.e., using repetitive extragenic palindromic (REP) or repetitive BOX target sequences), amplified fragment length polymorphism (AFLP) or multilocus restriction typing (MLRT) were applied with great success (Table 18.2). These investigations revealed existence of other epidemic strains; however, evaluation of their genetic relatedness between centers or countries was hampered by general limitations of all gel-based typing techniques: they are not easily transferable between laboratories and lack unambiguous criteria for interpretation of band pattern findings. These pitfalls have been recently overcome by introducing MLST into Bcc analyses.

MLST, a technique which is based on comparison of nucleotide sequences in seven housekeeping genes (*atpD*, *gltB*, *gyrB*, *recA*, *lepA*, *phaC* and *trpB*), was first employed for Bcc bacteria by Baldwin et al. in 2005 [9]. Since then, numerous Bcc isolates across the world were analyzed and information about their sequence type, or ST, deposited in the public MLST database (http://pubmlst.org/bcc/). Ease of data transfer, high reproducibility and precision of the method enabled to extend

Bcc strain comparisons beyond "a single lab analysis" and to define other than ET12 globally distributed epidemic strains [8, 10] (Table 18.2).

Apart from tracing of global distribution of Bcc, MLST has also proven to be a very suitable tool for local surveillance of infection. The working definition for a strain in MLST analysis, that is "one strain equals to one ST," facilitates easier interpretation of typing results for both microbiologists and clinicians who may have difficulties with interpreting data from gel-based typing methods. For example, the use of PFGE in Bcc epidemiology may produce results that are difficult to interpret as the method can generate multiple band variants for a single ST. From this standpoint, PFGE appears to be a method that is highly discriminatory and not as suitable for Bcc genotyping. In contrast, PCR fingerprinting methods appear to be reliable pattern-matching methods for analysis of genetic relatedness among clinical isolates. They can serve as a good alternative method to MLST whose application to every single isolate recovered from a single patient may not be absolutely necessary, and also because of its relatively high cost and a need for multiple sequencing (one isolate requires 14 sequencing reactions).

Besides strain analysis, MLST also allows easy identification of isolates to the species level. The study by Baldwin et al. [9] proved that concatenation and analysis of the seven MLST gene sequences for 114 Bcc isolates created phylogenetic tree clusters that corresponded with clusters by species. A few years later, Vanlaere et al. [3, 11] confirmed the excellent resolving power of MLST for species identification and revealed seven more distinct clusters for seven novel Bcc species. MLST typing of *Burkholderia* species beyond Bcc has also been recently achieved by Spilker et al. [12], who redesigned MLST primers to make them complementary to *Burkholderia gladioli*, *B. mallei* or *B. pseudomallei* (although an independent MLST scheme and database exists for the latter organism on http://bpseudomallei.mlst.net/). Today, information on what species a particular ST belongs to is provided in the public MLST database for most of submitted STs. Alternatively, it can be obtained from tree construction of concatenated nucleotide sequences of all available STs including the one in question. With successful application in many areas of bacterial analysis, MLST is becoming a key method not only for strain genotyping, but also for *Burkholderia* species determination.

18.4 Concluding Remarks

Correct identification of the Bcc organisms, which is usually achieved by *recA*-PCR, is absolutely essential from the standpoint of infection control in CF centers as well as epidemiological studies. Bacterial typing which follows Bcc detection can be performed either by methods where DNA fragments are separated on a gel (RAPD, rep-PCR, AFLP), or by sequence-based MLST which, as opposed to gel-based typing methods, also solves the question of Bcc species identification and produces reliable lab-to-lab comparisons. Because of these advantageous features we believe that MLST should be adopted as the gold standard for Bcc genotyping,

with the pattern matching methods used for analysis of local short-term epidemics or primary screening of large collections of isolates prior to full MLST work up of distinct strains.

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Part IV
Urogenital Pathogens

Chapter 19
Treponema

Allan Pillay

19.1 Introduction

The genus *Treponema* comprises a diverse group of organisms with varied ecological niches. Treponemes that are pathogenic for humans include the causative agents of venereal syphilis (*Treponema pallidum* subspecies *pallidum*), yaws (*T. pallidum* subsp. *pertenue*), endemic syphilis (*T. pallidum* subsp. *endemicum*), and pinta (*Treponema carateum*). In addition, some oral treponemes such as *Treponema denticola* are highly associated with periodontal disease. However, in terms of economic, health, and social impact, venereal syphilis outweighs any of those conditions caused by other pathogenic treponemes. *T. pallidum* is the only organism within the genus *Treponema* for which a typing system has been described; therefore, this chapter will focus mainly on *T. pallidum* subsp. *pallidum* (hereafter referred to as *T. pallidum*).

Venereal syphilis (hereafter referred to as syphilis) has a worldwide distribution. The disease is endemic in many developing countries and there has been a resurgence of the infection in some industrialized nations. In the United States, syphilis disproportionately affects some minority groups and, since 2001, data from the Centers for Disease Control [1] suggest that the increase in syphilis was largely a result of spread of the disease among men who have sex with men (MSM). A subsequent increase among the heterosexual population may be a problem in the near future. Since the 1940s, penicillin has been effective for the treatment of syphilis; however, the disease remains a significant public health problem, even in a number of industrialized

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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nations. Azithromycin has been used as an alternative to penicillin therapy in the United States; however, treatment failures associated with azithromycin-resistant *T. pallidum* strains have been documented [2, 3]. In the United States, past control efforts have resulted in a significant decline in syphilis rates; however, the disease remains a problem despite public health efforts to eliminate the disease.

The release of the annotated genome sequence of *T. pallidum* [4] and the development of a typing system [5] have provided important tools for studying the molecular epidemiology of syphilis. However, the lack of an in vitro cultivation system for *T. pallidum* and the inability to genetically manipulate the bacterium are obstacles to elucidation of virulence factors involved in the pathogenesis of *T. pallidum*. In addition, the complex natural history of syphilis and the stigma associated with the infection have made epidemiologic studies of this pathogen challenging.

19.1.1 Historical Perspective of Treponematoses

In the mid-twentieth century, yaws was prevalent in the tropics; endemic syphilis in Afghanistan, North Africa, southern Africa, south-west Asia, China and Europe; and pinta in Mexico, central and South America. Yaws control campaigns using penicillin aluminum monostearate and benzathine penicillin were very effective in reducing the prevalence of the active form of the disease in countries such as Haiti, Indonesia, and Jamaica. This led to the epidemiologic concept that penicillin treatment was necessary for asymptomatic household contacts and presumed latent cases in order to eliminate the reservoir of infection [6]. Soon after its establishment in 1948, the World Health Organization (WHO) initiated a worldwide campaign together with The United Nations Children's Fund to control yaws in 46 countries. Consequently, 50 million people were treated over two decades, resulting in a decrease in the prevalence of the disease by about 95%.

Compared to the endemic treponematoses, syphilis has clearly attracted more attention, in part, because of its mode of transmission, morbidity in adults, and sequelae in infants born to infected mothers. Syphilis rates peaked in western countries around World War II with a subsequent sharp decline in rates, which coincided with widespread use of penicillin for treatment. However, comprehensive data were only available in a few industrialized countries. In the United States, the reported incidence of primary and secondary syphilis peaked at just over 100/100,000 population in 1948. This was the highest rate reported since official notification began in 1941 [7]. In the United Kingdom, the incidence of primary and secondary syphilis was 4.56/100,000 population among men and 0.99/100,000 population among women in 1967, with a declining trend in overall syphilis rates being observed between 1950 and 1967 [8]. Syphilis trends in Canada were similar to those in the United Kingdom. Early infectious syphilis rates in the Netherlands began to decline in the 1980s from a peak of 61/100,000 in 1981 to 30/100,000 in 1985 [9]. In contrast, prevalence data, based on serology, from many developing countries in the 1970s and 1980s indicate an ongoing high prevalence of disease [7].

19.1.2 Current Epidemiology of Treponematoses

Current epidemiological data on the endemic treponematoses are either non-existent or difficult to obtain since most countries no longer collect data owing to the stigma of underdevelopment associated with these diseases [10]. Despite mass treatment campaigns conducted in the 1950s, the WHO estimates that about 2.5 million people are currently affected by the endemic treponematoses worldwide, with yaws accounting for the majority of cases [11]. A WHO initiative to eliminate yaws in India, Indonesia, and Timor-Leste by 2012 is currently underway and another campaign to eliminate yaws worldwide is currently being explored.

Based on 2005 prevalence estimates, approximately 10.6 million new cases of syphilis occur each year worldwide [12]. The majority of syphilis cases occur in sub-Saharan Africa, South and South-East Asia and Latin America and the Caribbean. Syphilis is endemic in many developing countries, while industrialized nations are characterized by low-level transmission and/or sporadic outbreaks. In the United States, syphilis rates were on the decline from 1990, when the crack cocaine epidemic reached a nadir; however, since 2000 the trend has reversed. The majority of syphilis cases have been reported among men, suggesting that the spread of the disease was occurring mainly among MSM. This increase in syphilis is characterized by high rates of human immunodeficiency virus (HIV) co-infection, high-risk sexual behaviors, and recreational drug use.

In addition to the increase in syphilis cases among MSM in the United States, there has been a resurgence of the disease among the heterosexual population, characterized by an increase of cases among women and infants. After a 5-year decline from 2001 to 2004, syphilis rates increased from 0.8/100,000 to 1.0/100,000 among women [1]. The rate of congenital syphilis increased from 8.2 to 8.5 cases per 100,000 live births between 2005 and 2006 after a 14-year decline [1]. Syphilis is also making a resurgence in countries such as Canada, the UK, Denmark, Belgium, Austria, Russia, and China [13]. In 1996, incident syphilis in the Russian Federation was alarmingly high with rates >900/100,000 population reported in men between the ages of 20 and 29 years [14]. During the past decade syphilis outbreaks in Europe and the United Kingdom have been attributed to both local and imported sources of the disease.

19.2 The Genus *Treponema*

The genus *Treponema* comprises a diverse group of organisms which are included among the commensal microbial flora of humans, animals, and insects. These organisms are also found in environmental sites such as contaminated aquifers. Bacterial species belonging to this genus are typically anaerobic, are host-associated, some of which are pathogenic for humans. Of the pathogenic treponemes, the etiologic agent of venereal syphilis (*T. pallidum*) is the most frequently studied by spirochetologists. Another two very closely related human pathogens, *T. pallidum* subsp.

pertenue and *T. pallidum* subsp. *endemicum,* are responsible for yaws and endemic syphilis, respectively. *T. carateum*, which causes pinta, while related to *T. pallidum*, was placed in a separate species owing to the lack of genetic information. *Treponema paraluiscuniculi* causes venereal syphilis in rabbits and is closely related to the etiologic agents of yaws, endemic syphilis, and venereal syphilis in humans [15, 16].

T. denticola, *Treponema vincentii*, *Treponema pectinovorum*, *Treponema medium*, *Treponema amylovorum*, *Treponema maltophilum*, and *Treponema socranski* are found in the oral cavity and have been shown to be associated with a shift from a healthy status to gingivitis and eventually to periodontitis [17]. *Treponema refringens* and *Treponema phagedenis* are commensals found in the genital tract of humans. Other spirochetes belonging to the genus *Treponema*, such as *Treponema succinifaciens*, *Treponema bryantii*, and *Treponema primitia,* have been isolated from the intestine of horses, rumen of cows, and the hindgut of termites, respectively. A number of as-yet unidentified oral and environmental treponemes have been detected by using 16S rRNA gene sequence analysis. Of the human-associated spirochetes, *T. refringens*, *T. phagedenis*, *T. denticola,* and a number of other oral spirochetes can be cultured on artificial culture media. The *T. pallidum* subspecies can be propagated through intratesticular inoculation of rabbit testes but has not yet been grown on artificial media.

Members of the genus *Treponema* have a unique cell structure and belong to the family Spirochaetaceae. The cell envelope of all treponemes consists of an outer membrane, a peptidoglycan layer, an inner membrane, and a characteristic number of flagellar filaments located in the periplasmic space. The shape of the organism is wavelike, helical, coiled, or serpentine, and treponemes exhibit a characteristic "corkscrew" motility in liquid media. Treponemal cells typically range in length from 5 to 20 μm and between 0.1 and 0.5 μm in diameter.

19.3 Clinical Diagnosis of *T. pallidum* Infections

T. pallidum subsp. *endemicum* (bejel), and subsp. p*ertenue* (yaws), like *T. pallidum* (syphilis), cause skin lesions and infections that can affect the skeletal system and other organs. However, compared to bejel and yaws, syphilis is a complex disease, exhibiting a variety of clinical manifestations. Syphilis can be divided into distinct stages: primary, secondary, latent, and tertiary syphilis (Fig. 19.1). Primary syphilis is usually characterized by the presence of a solitary chancre, a painless ulcer that begins in most instances as a papule at the site of inoculation; however, some patients do present with multiple lesions. Extragenital lesions have been reported to occur but are less frequent and tend to be painful compared to lesions in genital areas. The primary lesion usually appears 10–90 days postinfection with an average of 3 weeks. Typical primary syphilitic lesions appear in the genital area with bilateral inguinal lymph adenopathy observed in the majority of individuals. The disease is systemic following initial infection and 30% of untreated primary syphilis cases present with multiple secondary lesions of the skin or mucous membranes.

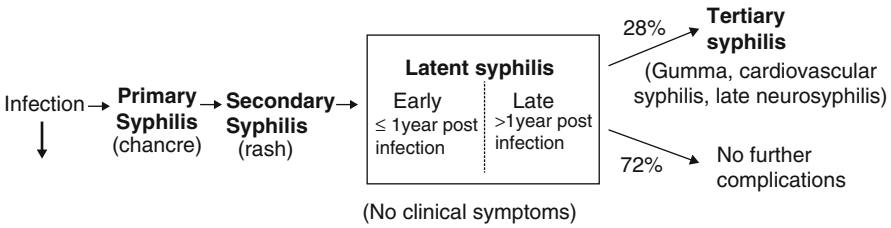

Fig. 19.1 Natural history of untreated syphilis, modified from Gjestland 1995

Secondary syphilis typically appears 6–8 weeks after the appearance of a primary chancre. It is characterized by evanescent macular rash followed by symmetric papular eruption, which occurs on the entire trunk and the extremities including the palms of the hand and soles of the feet. Condylomata lata, which are raised, whitish or grey lesions are frequently observed in warm, moist areas such as the vulva or perianal region. Other symptoms include generalized lymphadenopathy, fever or malaise, sore throat, headache, and weight loss [18]. In about 30% of patients, primary lesions are still present when secondary manifestations appear. *T. pallidum* invades the central nervous system (CNS) in at least 40% of secondary syphilis cases [19].

The secondary and tertiary stages are separated by a prolonged period of latency, which is characterized by a lack of clinical signs of disease. Latency occurs in patients without a history or serological findings consistent with syphilis and consists of early and late latent phases (Fig. 19.1). Early latent is the asymptomatic period between the spontaneous resolution of untreated secondary syphilis and tertiary period when the disease is less than 1 years' duration. Late latent syphilis is defined as latent syphilis of more than 1 years' duration.

Late or tertiary syphilis is divided into neurosyphilis, cardiovascular syphilis, and late benign syphilis. The signs and symptoms of tertiary syphilis usually occur many years after acquiring syphilis and any organ in the body can be affected. With the exception of neurosyphilis, late benign syphilis is relatively uncommon these days most probably as a result of inadvertent treatment of syphilis with treponemocidal antibiotics. In the preantibiotic era, about a third of patients with untreated syphilis developed tertiary disease, which manifested as neurosyphilis, gummas, or cardiovascular disease. Late neurosyphilis, which usually occurs in 15–20% of patients, presents as paresis, tabes dorsalis, or meningovascular syphilis; however, some patients may have asymptomatic disease with cerebrospinal fluid (CSF) abnormalities such as pleocytosis, elevated protein levels, and a positive Venereal Diseases Research Laboratory (VDRL)-CSF test. Diagnosis of tertiary syphilis is based on a combination of clinical history, serological tests, examination of chest radiographs for suspected cardiovascular syphilis, and long bone radiographs to detect bony gummas. Concomitant HIV infection has an impact on neurologic involvement in syphilis and several studies have documented the rapid progression from early syphilis to neurosyphilis characterized by meningitis or cranial nerve defects. In situations where the endemic treponematoses and venereal syphilis are prevalent, disease history, clinical presentation, and serological tests results are important considerations for patient management.

Congenital syphilis primarily occurs when *T. pallidum* enters the foetal circulation by transplacental passage from an infected mother. There is no primary stage of the disease since *T. pallidum* directly enters the foetal circulation and, consequently, the organism can infect any tissue in the body. Stillbirth is a frequent outcome of untreated maternal syphilis, accounting for up to 50% of congenital cases. Other conditions such as spontaneous abortion, premature delivery, and perinatal death are also associated with congenital syphilis. Clinical signs of congenital syphilis include hepatosplenomegaly, cutaneous lesions, osteochondritis, and snuffles. About 50% of infants with congenital syphilis are asymptomatic at birth; however, teeth and bone malformation, deafness, blindness, and learning disabilities may develop later. The CDC recommends the screening of women for syphilis during the early stages of pregnancy as a means to ensure effective prevention and detection of congenital syphilis.

19.4 Laboratory Diagnosis of Treponematoses

19.4.1 Direct Detection Methods

Compared to other infectious diseases, the laboratory diagnosis of syphilis poses a challenge, owing to the variety of clinical manifestations and, in some cases, the difficulty in obtaining adequate or appropriate specimens for testing. Treponemes cannot be observed with an ordinary light microscope due to their narrow width; therefore, a microscope equipped with a darkfield condenser is required. Darkfield microscopy (DF) can be used to detect *T. pallidum* in specimens from epidermal and mucosal lesions of primary, secondary, and early congenital syphilis with a reported sensitivity approaching 80% [20]. In addition, lymph node aspirates, neonatal nasal discharge, and amniotic fluid may contain enough spirochetes for successful examination by DF [21]. DF must be performed immediately upon specimen collection because it relies on observation of motile treponemes. DF is not recommended for oral lesions since the morphology of other treponemes such as *T. denticola* is indistinguishable from *T. pallidum*. The advantage of DF is that it can be performed in a peripheral clinic with a very short wait time. The direct fluorescent antibody test for *T. pallidum* (DFA-TP) test is specific for *T. pallidum* and can be used in place of DF for body fluids and lesion exudates; however, this test is seldom used because reagents are not readily available. The DFAT-TP is a modification of the DFA-TP and is intended for use on tissue specimens.

19.4.2 Polymerase Chain Reaction Tests

Several PCR assays, based on different gene targets, have been described for the detection of *T. pallidum* [22–25]. A commercial PCR assay is available (Sacace Biotechnology, Italy); however, this test has not been evaluated against other PCR assays and is not widely used. The multiplex PCR assay described by Orle et al. [23],

which detects *T. pallidum*, *Haemophilus ducreyi*, and Herpes simplex virus 1 and 2, has subsequently been modified into a quadriplex real-time PCR format (Chen CY unpublished data). The new assay offers the advantages of high-throughput testing and elimination of PCR contamination arising from post-PCR manipulation of amplicons. PCR is particularly useful for the establishment of a syphilis diagnosis in cases of genital ulcer disease and in some cases of secondary syphilis, where there are moist lesions. In addition, detection of *T. pallidum* by PCR in cases of suspected neurosyphilis, congenital syphilis, syphilitic gummas, and gastric lesions has also been documented. Since humoral antibodies only appear 1–4 weeks after the chancre has formed, direct detection methods such as DF, DFA-TP, and PCR are more sensitive than treponemal and non-treponemal serological tests in early primary syphilis. Although PCR-based tests are being increasingly used in cases where microscopic or serological methods fail due to a lack of sensitivity, there is no standardized PCR test for the detection of *T. pallidum*. The lack of standardization is due, in part, to the fact that it remains an emerging technology, having evolved from a conventional assay to one that can be performed in real time with a short turnaround time.

19.4.3 Serological Tests

Serological tests for syphilis are divided into treponemal and non-treponemal serological tests. Non-treponemal tests include the VDRL and rapid plasma reagin (RPR) tests, which detect anti-cardiolipin antibody in serum. The *T. pallidum* haemagglutination assay (TPHA), *T. pallidum* particle agglutination assay (TPPA), and enzyme immunosorbent assays (EIAs) are commonly used treponemal tests that detect antibody to surface exposed *T. pallidum* proteins. In the United States, screening for syphilis traditionally consists of testing sera with a non-treponemal test and reactive samples retested with a specific treponemal test. The fluorescent treponemal antibody absorption (FTA-ABS) test was popular in the past but less common these days because of its requirement for fluorescence microscopy and highly trained personnel. Pope et al. [26] demonstrated that the TPPA and Captia Syphilis-G EIA tests were appropriate substitutes for TPHA. Young et al. [27] reported that the TPPA test was significantly more sensitive than the FTA-ABS test and marginally more sensitive than the TPHA test, making TPPA a good confirmatory test. EIAs are being increasingly used in high-throughput laboratories and have been widely used for screening purposes as well as confirmatory testing owing to their high specificity, high sensitivity and amenability for automation.

After initial screening with a non-treponemal/treponemal test combination such as the VDRL/RPR test and TPPA or EIA test alone, different scenarios may result with testing [28]: (1) both treponemal and non-treponemal tests reactive; (2) a reactive treponemal test but negative non-treponemal test; (3) a reactive treponemal test with a negative or reactive non-treponemal test; and (4) non-reactive treponemal and non-treponemal tests. Reactive non-treponemal and treponemal tests most likely indicate untreated syphilis (or other treponemal disease) at any stage or treated late syphilis. A reactive treponemal test but negative non-treponemal test most likely indicates

treated early syphilis patients (primary, secondary, early latent) but can include those with early untreated primary syphilis. Sera in the third group are most likely from patients with a false-positive non-treponemal test. It is the only group where additional confirmatory testing is usually recommended [28]. If the first-line confirmatory test is negative and a second confirmatory treponemal and non-treponemal tests are negative, then the result can be reported as a false-positive screening test. If at least one additional confirmatory test is reactive, then this signifies a low level of treponemal antibodies, which could be the result of a treated or longstanding infection, or to an early primary infection. IgM EIA testing can distinguish between the two scenarios since detection of specific antitreponemal antibodies in patients with no history of recent treatment suggests active disease. The fourth and last scenario is most likely due to patients without syphilis; however, sera from patients with tertiary syphilis or those who have not seroconverted (incubating syphilis) can give negative results with both treponemal and non-treponemal tests. Delayed seroreactivity and false-negative serological results have been reported in patients coinfected with HIV; however, this is relatively uncommon and the vast majority of patients can be accurately and reliably diagnosed with serological tests [29].

Sischy et al. [30] reasoned that there was little value in performing confirmatory testing (TPHA, FTA-ABS, TPPA) on patients from high prevalence settings (RPR positivity rates ≥5%) since the false-positive RPR rate is very low in this group (0.02%). On the other hand, the authors recommend that confirmatory testing be performed in a low prevalence setting (RPR positivity rates <5%). In general, the quantitative RPR test is used to monitor the efficacy of treatment for syphilis since falling titers indicate successful treatment. Since the majority of cases that test positive by treponemal tests usually remain positive for the rest of their lives, treponemal tests cannot be used to monitor treatment. False-positive results can be observed with treponemal and non-treponemal tests due to autoimmune diseases, dermatologic diseases, cardiovascular disease, leprosy, endemic treponematoses, and due to technical errors. In addition, viral infections including HIV, tuberculosis, and other STDs may cause false-positive reactions in non-treponemal tests. Venereal syphilis cannot be differentiated from the non-venereal treponematoses on the basis of serology.

The VDRL-CSF test is the standard serological test for neurosyphilis; however, false-negative results may occur, in which case an FTA-ABS test on CSF is recommended. The latter test has a lower specificity than the VDRL-CSF test but is highly sensitive. Newer technologies such as a rapid point of care test that includes both treponemal and non-treponemal test formats in a single test are currently being evaluated. Rapid point-of-care tests are advantageous because they do not require a laboratory, specialized equipment, or highly trained personnel.

19.4.4 Molecular Typing of T. pallidum

Molecular typing has been performed on, *T. pallidum subsp. pallidum* strains but the method can be applied to other subspecies owing to the high degree of genetic relatedness. While the development of the typing system is considered as a breakthrough in

syphilis research, epidemiological studies which utilize this methodology are lacking. There are several reasons for the paucity of molecular epidemiological data on syphilis. Firstly, most primary chancres are typically located on the fourchette, labia, or cervix of women or the perianal area or within the rectum of MSM; therefore, syphilis infection often goes unnoticed until the appearance of secondary lesions. Secondly, spirochetes are generally found in low numbers within the blood, CSF, and healing primary ulcers and PCR testing of these specimens is lacking in sensitivity. Lastly, *T. pallidum* cannot be cultivated on artificial cell-free media and strain typing is therefore reliant upon direct amplification of DNA sequences from clinical material. Despite these challenges, the typing system has been used with various specimens (genital ulcers, blood, CSF, secondary lesions) to characterize strains obtained from both endemic and epidemic settings with genital ulcers being the primary source of specimens for typing.

Previous attempts to type the *T. pallidum* subspecies using phenotypic methods such as protein profiles [31] or lectin agglutination patterns [32] have been unsuccessful. In addition, an attempt to develop a serotyping system using monoclonal antibodies raised against *T. pallidum* subsp. *pallidum* failed to differentiate *T. pallidum* from subspecies *pertenue* [33]. Prior to the completion of the genomic sequence of the *T. pallidum* Nichols strain, attempts at intra- and inter-strain molecular differentiation of the three *T. pallidum* subspecies—*pallidum*, *pertenue*, and *endemicum*—only identified point mutations in several genetic loci, which were not feasible for use in strain typing [16, 34].

The first typing system for *T. pallidum* is a PCR-based method, described by Pillay et al. [5]. While this system is designed for the characterization of *T. pallidum* strains, data obtained during the development of this method suggested that it might be useful in differentiating subsp. *pertenue* and subsp. *endemicum* strains as well [35]. The typing system entails PCR-RFLP analysis of the treponemal repeat protein (*tpr*) genes and determination of the number of 60-bp repeats within the acidic repeat protein (*arp*) gene. A subtyping method, based on determination of the number of homonucleotide G tandem repeat within *tp0279,* has recently been added in an attempt to further differentiate strains since the majority of strains characterized to date fall into a single type, 14*d* [36]. A schematic diagram of the typing system is shown in Fig. 19.2.

The *tpr* genes comprise a 12-member multicopy gene family (*tprA* to *K*), which have been divided into three subfamilies, based on predicted amino acid homologies. The Tpr proteins are homologous to the *T. denticola* major sheath protein (Msp), which is surface located and has adhesion and porin functions [4, 37, 38]. Therefore, the *tpr* genes are believed to play a role in antigenic variation, which probably enables some *T. pallidum* bacteria to evade the immune system despite a specific immune response that clears most treponemes during primary and secondary syphilis.

PCR-RFLP analysis of the *tpr* genes entails amplification of the predicted variable region of the subfamily II genes (*tprE*, *G*, *J*) with primers that bind in the conserved 5′ and 3′ ends. An approximately 1.8 kb region of the *tprE*, *G*, and *J* genes is simultaneously amplified using a two-tube nested PCR employing primer pairs B1, 5′ACTGGCTCTGCCACACTTGA3′/A2, 5′CTACCAGGAGAGGGTGACGC3′ and

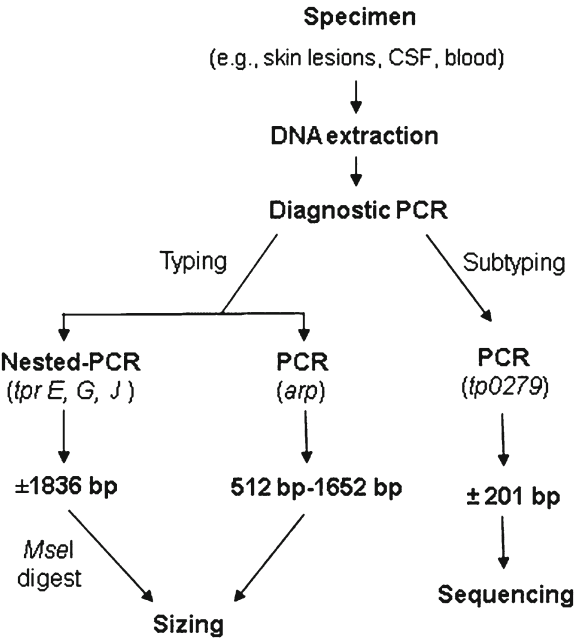

Fig. 19.2 Schematic diagram of the typing and subtyping methods of *T. pallidum*

IP6, 5′CAGGTTTTGCCGTTAAGC3′/IP7, 5′AATCAAGGGAGAATACCGTC3′ [5, 39]. The resulting amplicon is then digested with *Mse*I and restriction fragment sizes determined by agarose gel electrophoresis or by using an automated instrument such as an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) or Applied Biosystems 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Although restriction fragments can be separated on an agarose gel, using an automated instrument is advantageous particularly when new RFLP patterns are encountered. To date, 16 *Mse*I RFLP patterns, designated *a* to *p,* have been identified in *T. pallidum* strains [5, 39]. A schematic diagram of all the different RFLP patterns identified to date is shown in Fig. 19.3.

The 60-bp repeat region of the *arp* gene is amplified with PCR primers 1A (5′CAAGTCAGGACGGACTGTCCCTTGC3′) and 2A (5′GGTATCACC TGGG GATGCGCACG3′). The number of 60-bp repeats in the *arp* gene of *T. pallidum* strains varies from 3 to 22, corresponding to PCR amplicon sizes of 512–1,652 bp, respectively [39, 40]. Representative samples with different *arp* repeat sizes are shown in Fig. 19.4. The *arp* amplicon sizes can be determined as described for restriction fragments above. The advantage of using the Agilent 2100 Bioanalyzer is that no pre- or post-PCR labeling of primers or amplicons is required, while the use of ABI 310 genetic analyzer requires labeling of the forward or reverse primer with a fluorescent dye and incorporation of a fluorescent nucleotide during PCR amplification of the *tpr* genes. It is not necessary to use fluorescent dyes on both the

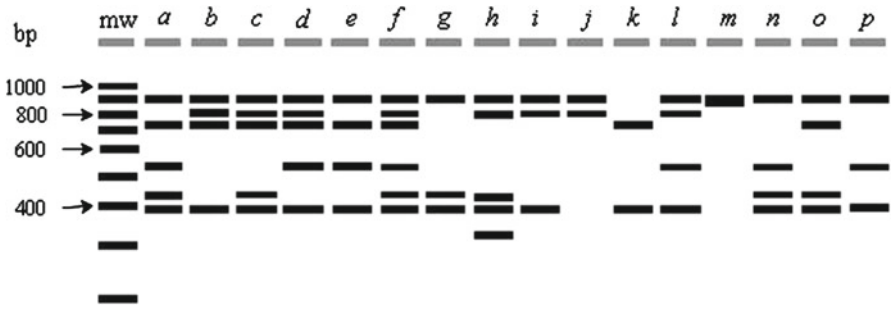

Fig. 19.3 Schematic representation of the different *Mse*I RFLP patterns identified to date with the *T. pallidum* typing system. Copyright statement—ASM Press

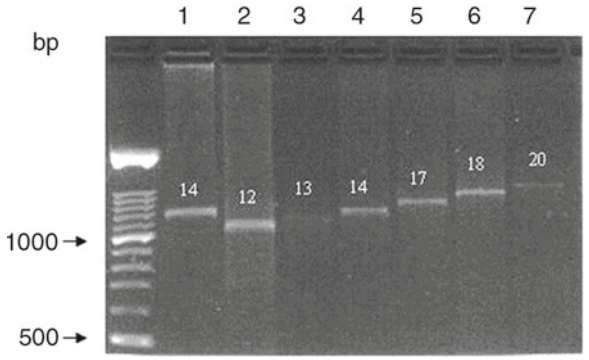

Fig. 19.4 Representative samples of the *arp* PCR showing different 60-bp repeat sizes. The number of repeats is shown *above* each band

forward and reverse primers as previously stated [5]. Labeling of either the forward or reverse primer with a fluorophore is adequate for sizing.

The third component of the typing system entails PCR amplification and sequencing of a homonucleotide G tandem repeat within the *tp0279* gene [41]. A strain subtype is designated based on a combination of the number of 60-bp repeats within the *arp* gene, the RFLP pattern of the *tpr* genes, and the number of G residues within *tp0279*. For example, a *T. pallidum* strain with subtype 14*d*9 has 14 60-bp repeats, the RFLP *d* profile and 9G repeats.

In the original paper describing the typing system [5], the authors characterized 38 clinical specimens obtained from patients with primary syphilis in two highly endemic areas (Madagascar and South Africa), the United States, and laboratory strains of *T. pallidum*. The authors demonstrated for the first time that different *T. pallidum* strains existed and that the typing method has the potential to be useful in epidemiologic studies on syphilis. The reproducibility of the typing scheme was further evaluated by typing samples of the Nichols strain of *T. pallidum*, which were obtained at different time points during rabbit passage over an 18-month period and

from in vitro culture over a 9-day period. Both the *arp* and *tpr* gene targets yielded reproducible results and thus were shown to be stable targets for use in typing. Pillay [35] typed specimens obtained from a woman who presented with a primary chancre and secondary skin rash and demonstrated the 14*i* strain type in both specimens. This further supports the stability of the gene targets used for typing. The finding, in several series, that the majority of strains belonged to a single strain type (14*d*) was not surprising considering the fact that the *T. pallidum* subspecies were believed to be a genetically homogenous group based on earlier studies. However, the authors attempted to further delineate the 14*d* strains by digesting *tpr* amplicons with additional restriction endonucleases with little success.

In a subsequent study, a cross-sectional analysis was performed to determine the diversity of *T. pallidum* strain types in three major cities and two mining towns in South Africa [39]. A total of 35 types were identified among 161 typeable specimens. This remains the most diverse collection of strains in a particular geographical area reported to date, although such diversity is likely to occur in other hyper endemic areas. The majority of strains 44/161 (27%) belonged to the 14*d* strain type and the number of types were directly proportional to the relative prevalence of syphilis. While the correlation was of borderline statistical significance ($p = 0.05$), it suggested that a high strain diversity reflected a high prevalence of syphilis.

Despite the complexity of syphilis and difficulties that are usually associated with obtaining an adequate specimen, the typing system has been successfully applied to specimens such as CSF [40] and blood [42, 43]. Sutton et al. [42] applied the typing system to genital ulcer and blood specimens obtained during an epidemiological investigation of a heterosexual outbreak of syphilis in Maricopa County, Arizona. Risk factors for syphilis transmission in that population were attributed to CSW (commercial sex work), sex with someone engaged in CSW, drug use, imprisonment, and homelessness. The 14*f* strain type was associated with white patients and the 12*a* strain type was identified in two epidemiologically linked cases. Recently, Florindo et al. [43] identified three couples that were epidemiologically linked but found discordant strain types in two of the three couples. To date, these are the only two published typing studies that have included specimens from epidemiologically linked cases. Additional studies are needed to determine if the typing system can correctly delineate strains from epidemiologically linked cases.

T. pallidum is known to invade the CNS in both early and late syphilis; however, patients with late neurosyphilis typically have fewer treponemes within the CSF. Molepo et al. [40] typed specimens from patients who were diagnosed as having late neurosyphilis based on clinical findings and VDRL and FTA-ABS test results. The authors reported that 56% (28/50) of CSF specimens tested positive using a diagnostic PCR targeting the 47 kDa gene of *T. pallidum* [23], of which 13 (46%) were typeable, yielding four strain types (2*i*, 3*e*, 14*a*, 17*e*). Of interest was the finding that 54% (7/13) of strains were type 14*a*, which suggests that this strain might have a tropism for the CNS. In a previous study, Pillay et al. [39] typed genital ulcer specimens from Johannesburg, which is in relatively close proximity of the hospital where the neurosyphilis typing study was conducted yet no 14*a* strains were identified in that study. However, Molepo et al. [40] point out that the neurosyphilis patients may have

acquired the infection in an area where the 14*a* strain was more prevalent. While this may be the case considering that the 14*a* strain was previously reported in Cape Town and Durban around the same time period, it is intriguing that all seven strains were identified in a single site involving a relatively small sample size. Moreover, the 14*d* strain, which was reported as being the predominant strain type in South Africa, was not detected in CSF specimens from the neurosyphilis cases [39].

Using a rabbit model, Tantalo et al. [44] demonstrated strain-specific differences in neuroinvasive capacity among six *T. pallidum* strains. Some strains were detected more frequently than others in CSF and did not show CSF pleocytosis, whereas other strains showed CSF pleocytosis but were detected infrequently in the CSF. One strain was negative for CSF pleocytosis or *T. pallidum* in the CSF. Further studies are warranted to determine if neurotropic strains exist in early or late syphilis and whether HIV coinfection increases the neuroinvasiveness of these strains.

Florindo et al. [43] characterized *T. pallidum* strains from patients with early syphilis in Lisbon, Portugal. Of 42 typeable specimens, three strain types were identified. Strain type 14*a* constituted 50% of specimens, 14*d* and 14*f* accounted for 45 and 4.8%, respectively. The 14*a* strain type was found in twice as many blood specimens compared to primary and secondary lesions while 14*d* was found almost exclusively in primary and secondary lesions. The authors stated that their inability to type 34% (12/35) and 43% (3/7) of primary and secondary syphilis specimens, respectively, was due to the poor sensitivity of the *arp* PCR. Previous findings [39, 42, 45] concur with Florindo et al. [43] that the *arp* PCR is less sensitive than the *tpr* assay; however, the percentage of typeable specimens were much higher than those reported by Florindo et al. [43]. The *arp* PCR was designed to amplify a broad range of 60-bp repeats ranging in size from 6 to 22 repeats. Since the majority of strains typed to date have 14 repeats, optimization of this PCR to amplify repeat regions containing up to 12–15 60-bp repeats should improve the sensitivity of the assay. Alternatively, designing PCR primers that amplify a smaller region or using a nested PCR should improve the sensitivity of the *arp* assay.

Pillay et al. [36] investigated the possibility of being able to further delineate 14*d* strains since the majority of strains belong to this strain type. They typed specimens from Cape Town, South Africa, a syphilis-endemic area and an outbreak setting in Vancouver, Canada. Interestingly, the 14*d* strains from the syphilis-endemic area had four different subtypes (8 to 11Gs) while all except one strain from the initial outbreak in Vancouver had 9Gs, suggesting that the 14*d*9 subtype might have been responsible for the outbreak. Katz et al. [41] applied the typing system to specimens obtained from patients with primary and secondary syphilis in San Francisco, where clinical failures due to azithromycin resistance in *T. pallidum* had been recently reported. The majority of strains were identified as 14*d*9; however, azithromycin resistance was not associated with a specific strain type. The lack of association between azithromycin resistance and a specific strain subtype in San Francisco is surprising considering that this study was conducted in a geographically defined area where the disease has a low level of endemicity and is largely confined to a specific risk group. However, this may not be a reflection of other areas such as those where syphilis is even less common and azithromycin resistance has been noted.

The typing system described by Pillay et al. [5] can be applied to different types of clinical specimens depending on the stage of syphilis. The method is labor intensive because it entails several PCR amplifications, RFLP analysis and sequencing; however, it has been shown to be useful for epidemiogical studies on syphilis.

19.5 Conclusion and Future Perspectives

The study of the pathogenic *Treponema*, particularly the etiologic agent of venereal syphilis, remains a major challenge. From an epidemiological standpoint, the stigma of having an STD and the complex nature of syphilis is an obstacle to studying the epidemiology of syphilis. Although some progress has been made to unravel the molecular epidemiology of syphilis, there are still a number of unanswered questions. Firstly, are syphilis outbreaks due to strains with a single *T. pallidum* strain subtype in a given setting or to a limited number of strains? Secondly, what is the pattern of sexual networking that enables the infecting strain to spread? Thirdly, do outbreaks occur as a result of strains already present in a given community or to the introduction of new strains? Fourthly, can the typing system be used to identify links between index cases and their contacts based on *T. pallidum* strain subtype? Lastly, is disease progression from primary to secondary stage strain related? In other words, are some patients more prone to develop secondary syphilis due to virulence of the infecting strain. Undoubtedly, many advances have been made to improve our understanding of the interaction of *T. pallidum* with its human host. The availability of the complete genome sequence of two *T. pallidum* strains hopefully will help to refine our tools for studying the molecular epidemiology of syphilis and ultimately controlling the disease.

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Chapter 20
Chlamydiaceae

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20.1 History and Clinical Relevance

The order Chlamydiales comprises four families, including *Chlamydiaceae*, and contains a single genus, *Chlamydia*. There are currently nine species of *Chlamydia,* and only three are known to infect humans: *Chlamydia trachomatis*, *Chlamydia pneumoniae,* and *Chlamydia psittaci*. *C. trachomatis* is a globally important pathogen, since it is the leading cause of preventable blindness and bacterial sexually transmitted infections (STI) in the world today [1–3]. *C. pneumoniae* is responsible for upper and lower respiratory tract infections worldwide and has been implicated in atherosclerosis [4], ischemic strokes [5, 6], and asthma [7]. *C. psittaci* is likely widely distributed among susceptible avian and mammalian species; humans are an accidental host, acquiring ocular and pulmonary infection from contact with infected secretions, feces or tissue [8]. These infections are referred to as psittacosis, ornithosis, or parrot fever.

Unique to Gram-negative *Chlamydiaceae* are their obligate intracellular nature where they exist in biphasic forms. The infectious, metabolically inactive form is referred to as an elementary body (EB), while the metabolically active form is referred

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to as the reticulate body (RB). The EB transforms into an RB shortly after entry into the host cell with subsequent formation of an intracytoplasmic inclusion body. Ineffective host lysosomal fusion ensures survival of the inclusion body for the duration of replication by binary fission, which proceeds from ~24 to 72 h depending on the species and strain type. Disruption of this developmental cycle likely occurs from amino acid deprivation, penicillin, or other types of treatment, which can lead to persistent ocular or sexually transmitted infections [9, 10].

C. trachomatis is divided into two biological variants (biovars): trachoma and lymphogranuloma venereum (LGV). The biovars are further subdivided into serologically defined groups based on antibody recognition of variant antigens on the major outer membrane protein (MOMP) of the microorganism. Serogroup B comprises serological variants (serovars) B, Ba, D, Da, E, L_2, L_2a, and L_2b. The intermediate serogroup comprises serovars F and G, and serogroup C comprises serovars A, C, H, I, Ia, J, Ja, K, L_1 and L_3. Serovars A, B, Ba, and C are the predominant causes of the ocular disease referred to as trachoma, which can lead to chronic visual deficits and blindness. Both serovars Ba and C can also cause urogenital infections, although infrequently. Serovars D to K are primarily responsible for genitoanalrectal infections and unilateral conjunctivitis. Approximately 80% of the urogenital infections are asymptomatic among women, while 50% are asymptomatic among male populations [11]. Up to 40% of untreated women develop pelvic inflammatory disease (PID) that can lead to the complications of infertility, chronic pelvic pain, and ectopic pregnancy [12]. An untreated pregnant woman presents an increased risk of ophthalmia neonatorum and pneumonitis for her newborn infant. The LGV strains, L_{1-3}, L_2a, and L_2b, are more invasive, causing genital ulcers, inguinal lymphadenitis with or without suppuration, hemorrhagic proctitis, and rectal strictures. These infections require a much longer treatment duration than uncomplicated chlamydial infections [13].

There are distinct limitations to the detection and strain typing for each of the three species. For *C. trachomatis*, the primary detection methods include commercial nucleic acid amplification tests (NAAT) and probes. The drawbacks are the cost, required technical expertise, need for specialized equipment, and time (up to a week) before the test result is available. In addition, these tests are unable to discriminate invasive LGV strains from the other ocular or genitourinary strains, which is important for treatment decisions as mentioned above. Strain typing is confined to epidemiologic and research studies limiting their clinical application. For *C. pneumoniae*, serology and culture of the nasopharynx, sputum or pleural fluid are important in determining a diagnosis but are neither routinely available nor approved by the Federal Drug Administration (FDA) [14]. NAATs are being developed for sputum but have the same limitations as listed above. Strain typing is not performed. For *C. psittaci*, serology is primarily used for making a diagnosis, but is not FDA approved [8]. Culture is problematic because of the highly virulent nature of this pathogen and is confined to reference laboratories. Serotyping using a battery of antibodies is available in reference laboratories [15] to differentiate strain types that may be more important in human versus animal diseases. In addition, a newly developed array tube technology has been shown to detect all *Chlamydia* species from

Table 20.1 Assessing the relevance and application of different chlamydial assays according to professional occupations

	Clinician	Epidemiologist	Research scientist
Serological diagnostics			
MIF/WIF	++	+	–
CF	++	+	–
EIA	++	+	–
Commercial ELISA	–	+	–
Chlamydial antigen ELISA	–	+	+++
Direct detection of chlamydial bacteria			
Cell culture	+	+	+
DFA	++	+	–
Serotyping	+	++	+
Nucleic Acid Amplification Tests (NAAT) for detecting DNA or RNA			
LCR and PCR	+++	++	++
Real-time PCR	+++	++	++
RDB and RLB	+	+++	+
Strain typing techniques			
DNA nuclear probes	++	+	+
ompA sequencing	+	+++	++
RFLP	–	++	+
AFLP	–	+++	++
Microarrays	+	+++	+++
MLST	–	+++	++
Genomic sequencing	–	++	+++
Point-of-care testing	+++	+++	+

amplified DNA [16, 17]. Table 20.1 provides a summary of the various diagnostic tests and strain typing tools that are either currently available or emerging as alternatives for clinicians, epidemiologists, and research scientists. Each of these is covered in the following text.

20.2 Serologic Diagnostics

20.2.1 Complement Fixation and Microimmunofluorescence

Microimmunofluorescence (MIF) and/or complement fixation (CF) tests are used to detect IgM or IgG antibodies against *Chlamydia* species in a single serum specimen (IgM titer of ≥1:16) or a fourfold rise in IgG antibodies in paired sera collected 2 weeks apart [11]. These tests, both commercial and in-house, are primarily

used for diagnosing acute *C. pneumoniae* or *C. psittaci* infections and are considered the gold standard, although they are not FDA approved. In some cases, these tests are used to screen women for evidence of exposure to *C. trachomatis* STIs, but cross-reactivity among the different species limits the utility of these tests. Recent studies have shown that commercial ELISA tests for IgG and IgA (CT-EIA, Labsystems; SeroCT, Savyon Diagnostics Ltd., and CT pELISA, Medac) have similar or improved sensitivity, specificity, and positive and negative predictive values compared to MIF [18]. The use of such methodologies to assess possible chronic versus acute infections, previous exposure, and characterization of protective and pathologic inflammatory responses suggest these techniques deserve further evaluation.

Complement fixation is a technically demanding serological test developed in 1935. This test is only genus-specific for the lipopolysaccharide (LPS) antigen of *Chlamydia* and has limited use for diagnosing *C. psittaci* and LGV. Briefly, chlamydial LPS is mixed with guinea pig complement, sheep RBCs, and patient samples (i.e., serum, bronchoalveolar lavage, etc.). A single positive titer of ≥256 demonstrated by the absence of RBC lysis is considered positive for LGV. Serum CF titers of ≥64 are considered positive for any *Chlamydia* species.

For MIF, EBs from all serovars of *C. trachomatis* and one or two strains of *C. pneumoniae* and *C. psittaci* are fixed onto glass slides. Biological samples (i.e., tears, serum, or cervical and other bodily fluids) are applied as serial, two-fold dilutions with subsequent addition of a fluorescein-conjugated secondary antibody. Observation of a number of green fluorescently labeled EBs is interpreted as a positive test. This serological technique is considered more efficient and accurate than CF as demonstrated by a study that compared both methods for diagnosing human psittacosis [19]. A similar test, Whole Cell Inclusion Immunofluorescence (WIF), differs slightly from MIF in that the entirety of the inclusion body is used as an antigen, unlike the EB alone as in MIF [20]. This allows both genus-specific (e.g., LPS) and species-specific (e.g., *C. trachomatis* MOMP) antigens to be detected, albeit in the same sample. Additionally, antigens expressed on RBs may also be recognized and may have utility in detecting chronic, persistent infections that may occur in the upper reproductive tract among women with infertility [21]. Identification of tissue-specific host antibodies to EBs by MIF has led to the association of *C. trachomatis* infections with Reiter's syndrome [22], salpingitis [23], tubal factor subfertility [24], and trachoma [25]. Additionally, epidemiologic studies have provided evidence for *C. pneumoniae* antibodies that suggest a role in pneumonia [26], atherosclerosis, ischemic strokes [5], and trachoma [25]. However, the use of MIF in detecting clinical *C. pneumoniae* infections is uncertain, considering that approximately 60% of adults over the age of 30 years have antibody titers against *C. pneumoniae* [27]. Additionally, the technique requires a high level of technical expertise, a fluorescent microscope, and the necessary EBbs of the various *Chlamydia* strains and species for application to the slides. Thus, this test is more restricted to reference laboratories.

20.2.2 Enzyme Immunoassay and EIA-PCR

Enzyme immunoassay (EIA) was among the first commercial tests available to assess the epidemiological presence of chlamydial infections in different populations and has the advantage of being less expensive and time consuming than MIF [18]. These tests have primarily been used to detect *C. trachomatis* STIs [21], but EIA has also been used to detect *C. pneumoniae* respiratory infections [28] especially with the advent of using species-specific LPS antigen [29] instead of whole EBs [30] as the capture antigen. Basically, a chlamydial antigen (i.e., *Chlamydia* LPS) is fixed to the bottom of 96-well plates. Patient samples are tested for immunoglobulin antigen recognition and detected by an enzyme-linked secondary antibody with subsequent addition of a substrate. The resulting colorimetric products are then measured by a spectrophotometer. This method is fairly quick (~3 h), easy to perform, and results in relatively high specificity (~98%). However, the sensitivity can be low depending on the commercial kit and capture antigen being utilized. Additionally, previous infections might also lead to false-positive results.

A slight modification to the EIA involving the addition of polymerase chain reaction (PCR) has improved clinical applications. Briefly, PCR is used to amplify chlamydial DNA (e.g., *ompA* gene) from clinical specimens. A biotinylated RNA probe specific to the PCR amplified DNA segment is then hybridized to the PCR product. Recognition of this RNA:DNA hybrid is performed with an enzyme conjugated Fab' fragment of a monoclonal antibody. Further steps mimic the EIA test where a substrate is added and the colorimetric product is read via spectrophotometry. This technique has been used to detect *C. trachomatis* in endocervical [31] and ocular [32] samples, as well as *C. psittaci* in respiratory samples [33]. Advantages include a semi-quantitative method, which can be used in epidemiologic studies and to assess clinical responses to anti-microbial therapy. Currently, the increase in whole chlamydial genome sequencing may provide insight into unique DNA sequences that could be used for detecting species and strains.

20.2.3 Enzyme-Linked Immunosorbent Assay

The development of an enzyme-linked immunosorbent assay (ELISA) has provided a more sensitive [34] and easier to perform technique for the detection of antibody titers against *Chlamydia* in patient biological fluids. This assay is similar to the MIF test in that chlamydial antigens are fixed to a surface, and serial diluents of biological samples are then tested. The major difference is the addition of an enzyme-conjugate [i.e., horseradish peroxidase (HR) or alkaline phosphatase (AP)] to the secondary antibody directed against human antibody (IgG, IgM, or IgA) followed by the addition of a substrate resulting in a colorimetric product. The results can be interpreted subjectively or quantified by absorbance using a spectrophotometer.

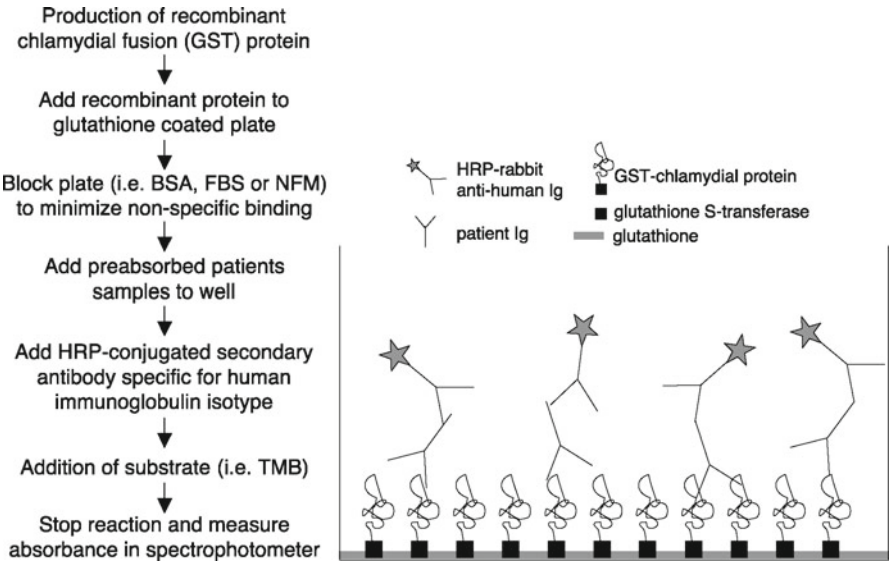
Production of recombinant chlamydial fusion (GST) protein
Add recombinant protein to glutathione coated plate
Block plate (i.e. BSA, FBS or NFM) to minimize non-specific binding
Add preabsorbed patients samples to well
Add HRP-conjugated secondary antibody specific for human immunoglobulin isotype
Addition of substrate (i.e. TMB)
Stop reaction and measure absorbance in spectrophotometer
HRP-rabbit anti-human Ig
patient Ig
GST-chlamydial protein
glutathione S-transferase
glutathione

Fig. 20.1 Schematic diagram of an ELISA used as a diagnostic tool for *Chlamydiaceae* species

The use of ELISAs to detect and quantify patient antibody titers from different anatomical locations against various chlamydial proteins allows characterization of different immunoglobulins for different clinical stages of disease. To date, the higher prevalence of serum and conjunctival IgG immunoreactivity against chlamydial heat shock protein 60 (cHSP60) compared to MOMP in patients with PID, atherosclerosis, and trachomatous trichiasis, respectively, have suggested that antibody titers against cHSP60 indicate a risk factor for chronic disease [35–37]. Characterization of other chlamydial-associated proteins in association with various stages of disease is required to more fully understand the association of antibody titers with disease risk and progression. Additionally, subclass characterization of IgG and IgA immunoglobulins is lacking which may lead to misinterpreting the results. Since IgG2a is a marker for T helper (Th) 1 type immunity whereas IgG1 is associated with a Th2 response [38], determining the immunoglobulin subclass could aid in differentiating between protective and immunopathogenic antibody associations.

Commercial ELISAs and in-house ELISAs that employ chlamydial fusion proteins representing known as well as new immunogens have served as diagnostic tools for identifying antibody titers in samples from different anatomic locations (Fig. 20.1). Mass screening of over 150 chlamydial proteins using recombinant GST-fusion chlamydial proteins was able to identify five new immunogens (CT089, CT147, CT694, CT226 and CT795) that elicit antibody responses in humans [39]. However, their pathologic or protective roles during acute and chronic diseases remain to be elucidated. Yet, one study showed a significant elevation in IgG antibodies levels to cHSP60, CPAF, and CT795 among patients with inflammatory

trachoma compared with controls; only elevated antibodies to CPAF were significantly associated with trachomatous trichiasis patients [40]. A few *in vitro* studies have demonstrated neutralization properties against *C. trachomatis* from monoclonals directed against MOMP and PmpD, though not for LPS [41]. However, the epitope recognized by these antibodies remains ill defined, rising questions as to whether the isotype, subclass, or antigenic region is the major determinant for these neutralization properties.

A few studies have utilized indirect ELISAs to demonstrate a higher frequency of host serum IgG and IgA against the P5 epitope of phospholipase D in *C. pneumoniae* among patients with acute coronary syndrome compared with healthy blood donors [42]. Additionally, use of a monoclonal antibody against a unique tetrasaccharide (3-deoxy -alpha-d-manno-oct-2-ulopyranosonic acid) on *C. psittaci* in the indirect ELISA was able to distinguish *C. psittaci* from *C. pneumonia* and *C. trachomatis* [43].

Future studies are needed to associate antibody isotypes and subclasses against various immunogens with protection versus immunopathogenic host responses. These assays are best suited to epidemiologic and basic research studies.

20.2.4 Leukocyte Esterase Test

The Leukocyte Esterase Test (LET) detects esterase release by polymorphonuclear leukocytes from first catch urine samples. Patients usually prefer this approach to invasive endocervical or urethral swab sampling. However, there is a wide range of documented specificities and sensitivities that make this assay unreliable unless it is used along with smears for detecting the number of leukocytes present in the urine [44].

20.3 Direct Detection of *Chlamydia* Species

20.3.1 Cell Culture

Cell culture has been the gold standard for detection of *C. trachomatis* due to the exceedingly high specificity (~100%), although it has a low sensitivity (70–85%) relative to commercial or in-house nucleic acid amplification tests (NAATs) even when performed by experienced personnel [45]. The benefits of cell culture include the high specificity and availability of isolates of the organism that can then be used for future molecular analysis or research studies. However, the long time required to propagate the organisms (3–7 days for detection and longer if isolates are to be stored for future use), the need for well-trained technicians, and the expense of cells, reagents, and disposables for culturing makes this technique more of a research tool than practical for clinical or epidemiologic studies. Typically *C. trachomatis* clinical

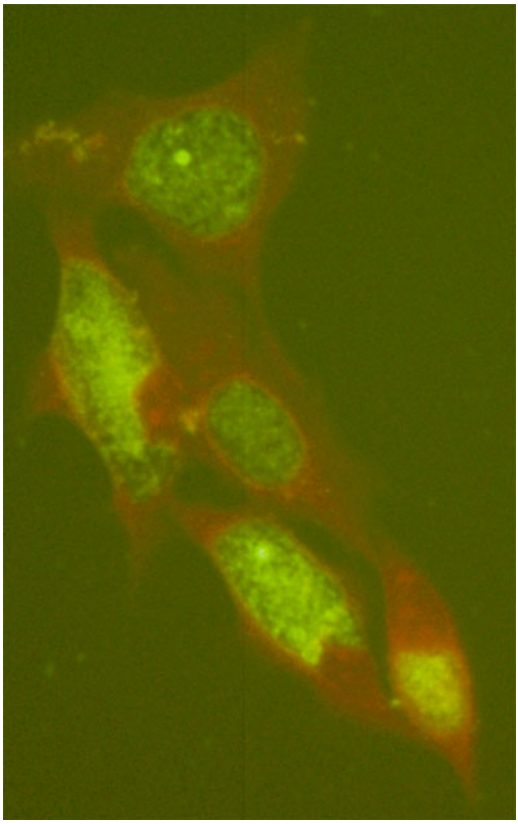

Fig. 20.2 Example of chlamydial inclusions that have formed in vitro within HeLa 229 cells. Cells are stained *red* with *Evans blue* and the inclusions are stained with a FITC conjugated monoclonal antibody directed against LPS. ×1,000 magnification

specimens are inoculated onto cell culture monolayers (i.e., HeLa or McCoy cells) in glass vials, multi-well culture plates, or varying sizes of tissue culture flasks for propagation and detection. *C. pneumoniae* and *C. psittaci* are usually grown in HEp-2, HUVEC, McCoy, or Vero cells. After 48–96 h of infection, the cells are fixed and stained with a FITC-conjugated monoclonal antibody with specificity against MOMP (species-specific) or LPS (genus-specific) (Fig. 20.2). This labor-intensive technique has been mostly replaced by NAATs described below. However, isolation of *Chlamydia* from clinical samples is imperative to further elucidate the biological relevance of different chlamydial strains in disease pathogenesis.

20.3.2 Direct Fluorescent Antibody

One of the first molecular assays utilized to detect *C. trachomatis* was the direct fluorescent antibody (DFA) test. DFA tests were very attractive due to their relatively rapid turnaround time and high specificity for detecting *C. trachomatis* in

ocular and endocervical samples [45] as well as *C. pneumoniae* and *C. psittaci* in ocular samples [46, 47]. Briefly, a swab is taken from the endocervix or conjunctivae, which is then rolled across a glass slide, fixed with methanol and stained with a fluorescent-conjugated monoclonal antibody against MOMP to detect fluorescing EBs. Some kits have used LPS as a marker. The latter is genus-specific and cross-reacts with most *Chlamydiaceae* organisms. However, staining with a monoclonal antibody against MOMP provides higher specificity and is cheaper than cell culture. The down side is the need for technical expertise and a fluorescent microscope, and the limited sensitivity due to false positives and the variability in interpretation as to the number of EBs required for a positive result with no inter-lab standardization. Thus, the test is limited to reference labs. Currently, the DFA test is rarely used given the plethora of other options discussed below.

20.4 Nucleic Acid Probes and Amplification Tests for Detecting *Chlamydia* DNA and RNA

20.4.1 DNA Nuclear Probes

An assay to detect the cryptic plasmid of *C. trachomatis* was developed with the hopes of increasing sensitivity and specificity and necessitating less stringent sample handling conditions. Cell culture and DFA rely on the presence of multiple inclusion bodies and EBs, respectively, in order to consider a sample positive for infection. However, in theory, detection of chlamydial DNA should only require one EB, since the cryptic plasmid is present in 7–10 copies. Early DNA probe experiments isolated cryptic plasmid DNA from *C. trachomatis*, digested it with restriction endonucleases, biotinylated the fragments, and hybridized the DNA to a nitrocellulose filter containing material from a conjunctival or endocervical swab [48]. If chlamydial DNA is present, the probe is detected by a streptavidin-alkaline phosphatase conjugate with addition of a BCIP/NBT substrate [49]. Although demonstrating strong positive and negative predictive values of approximately 96.5 and 97.5%, about 10 years later, a modified protocol was developed using amplified *C. trachomatis* rRNA and Southern blot technology to detect the organism (Amp-CT). This methodology alone increased the number of target copies 100-fold compared to a DNA sample alone and thereby increased the sensitivity levels above cell culture while retaining a high level of specificity [50].

Due to the need for trained personnel and the excessive time involved in both the PCR and subsequent Southern blot analysis, clinical applications dwindled and have been replaced by newer NAAT technologies discussed below. The use of nuclear probes would fail to detect SNPs or minute insertions and deletions within associated microbial genes. Detection of multiple strains is also not possible, suggesting minimal benefits to both epidemiologists and research scientists. Additionally, recognition of clinical strains lacking the cryptic plasmid [51] suggests it is imperative to identify essential genes specific to the genus and species.

20.4.2 Commercial Polymerase Chain Reaction

NAATs have significantly increased the sensitivity (80–97%) for detecting *Chlamydia* with a relatively high specificity (91–99%) over culture and DFA. Many different commercial tests for *C. trachomatis* are available, including Amplicor PCR Assay (Roche); APTIMA Combo 2 and APTIMA assay (Gen-Probe); Hybrid Capture 2 CT-ID DNA Test (Digene); and ProbeTec ET (Beckin Dickenson) [52–54]. However, the concordance in sensitivity between NAATs for detecting *C. trachomatis* in the same sample can be as low as 71.5% [55]. The tests are designed to detect one or two loci (*ompA*, 16S rRNA or the multi-copy cryptic plasmid) at a level of detection of 1–10 copies [56]. Recent studies demonstrated a deletion of 377 base pairs of DNA in the cryptic plasmid in clinical isolates from Sweden at the site where primers were designed for PCR amplification for both the Roche and BD tests [51, 57], suggesting that single locus detection systems are not and will not be reliable. Additionally, mutations arising at primer binding sites might also lead to false-negative results. There are currently no commercially available NAATs for *C. pneumoniae* or *C. psittaci*. Currently, none of these tests can detect strain type. With the increasing worldwide prevalence of LGV strains [58–60], this becomes an important issue since detection of LGV would necessitate a longer treatment interval for cure than non-LGV strains [61]. The additional down side of these tests is the requirement for a clinical lab that can batch process samples, the technical expertise required to run the assays even though they are kit-based, and the cost, all of which limits more widespread screening for these infections.

20.4.3 Commercial and In-House PCR and Real-Time (RT)-PCR

This method is very similar to the above PCR, but the length of the amplicon varies and is substantially smaller for RT-PCR, around 70–150 base pairs. In-house PCR has been described for all three species [9, 10, 14, 47]. Increased sensitivity of RT-PCR compared to conventional PCR and nested PCR was evident in detecting *C. pneumoniae* in atherosclerotic plaques [62] and oropharyngeal swabs from ischemic stroke patients, respectively [63]. Additionally, amplification of complimentary (c)DNA from conjunctival swabs from Nepali patients via RT-PCR for species-specific16S rRNA and MOMP resulted in identifying *C. psittaci, C. pneumonia,* and *C. trachomatis* as all etiologic agents of trachoma [64]. This technique significantly shortens the time period needed to assess a positive reaction without the use of an agarose gel. Other benefits include the quickness, minimal skills needed, and amplification of multiple genes to determine genus and species, thus making this appealing from a clinical and epidemiologic perspective. Elucidating microbial genomic expression of novel and putative genes also aids research scientists in elucidating chlamydial pathogenesis. However, the need to use SYBR Green and/or probes and a real-time PCR machine make it quite expensive. Additionally, the same problems are evident if mutations arise at sites where the

primers or probes sit down on the DNA, and a mixed infection with different serovars would be indistinguishable.

An alternative approach to this methodology would be to isolate RNA from the clinical sample, reverse transcribe and use the cDNA in RT-PCR. These results are more likely to identify active infection. However, it is crucial that specimens are assayed rapidly or stored appropriately due to the rapid degradation of RNA that might result in false negatives.

20.5 Strain Typing Techniques

20.5.1 Serotyping to Determine Strain Type

For the methodologies discussed so far, the current diagnostic tools are only able to discriminate among species of *Chlamydiaceae*. The isolation of *C. trachomatis* organisms from different anatomic sites led to the development of serological techniques to differentiate strain types. Polyclonal antibodies were the first to be developed against the MOMP of *C. trachomatis* isolates and subsequently were expanded to monoclonal antibodies to discriminate among serovars A, B, Ba, C, D, E, F, G, H, I, J, K, L_1, L_2, and L_3 including Da, Ia, and L_2a [65, 66]. The diversity of MOMP is evident in its four highly variable segments (VS1-4) with VS3 displaying the least diversity [67]. Epitope mapping has been able to pinpoint antigenic regions among the VSs, which define the specificity of the monoclonal antibodies [68]. Serotyping was also able to identify six serovars for *C. psittaci*: A, B, C, D, E, and F, and a new serovar E/B [69]. Some *C. psittaci* serovars have been noted to be endemic to different avian species localized to various geographical regions and associated with zoonotic transmission to humans (A, C, D, and E). However, to date, *C. pneumoniae* has not been differentiated into serovars or strain types, although variability within different genes of the micro organism has been noted [70]. The disadvantages of this technology include the need for culturing the organism, multiple different monoclonal antibodies and a fluorescent microscope. Propagation of the clinical samples prior to monoclonal typing requires expert personnel, a substantial time commitment, expensive reagents, and appropriate equipment. Serotyping is confined to reference labs but has been used for clinical or epidemiologic purposes. Researchers tend to rely on genetically based typing methods described below.

20.5.2 Reverse Dot or Line Blot Analysis (RDB or RLB)

Due to the exceedingly high price of monoclonal antibodies to detect serovar specificity, the difficulty and laborious work required for culturing clinical samples, and the lack of efficiency in detecting multiple infections, a new genomic method

was needed. Modification of the above procedures was combined to identify different serovars associated with clinical *C. trachomatis* infections. In RLB, the *ompA* gene is PCR amplified and the product further amplified via nested PCR using biotinylated primers. Oligonucleotide probes designed with 5′-amino terminal groups are then bound to a negatively charged membrane. After subsequent hybridization of the nested PCR products with probed membranes, biotinylation is detected using peroxidase-labeled streptavidin with subsequent addition of a chemiluminescent substrate. In one study, VS2 contained enough heterogeneity to allow serovar-specific probes to discriminate among 15 different serovars (E I, B, J, K, H, G, and F) [71]. This method was unable to discriminate all serovars (i.e., B and Ba, D and Da, and G and Ga), but was able to identify coinfections with multiple serovars. However, applications using these methodologies to detect *C. psittaci* and *C. pneumoniae* are rare. In one study that used RLB to identify *C. pneumoniae* in vascular disease, there were multiple false positives associated with contaminated water [72]. This finding reemphasizes the vital need to perform all of the proper negative controls to ensure adequate confidence in the results.

20.5.3 Restriction Fragment and Amplified Fragment Length Polymorphisms (RFLP and AFLP)

An alternative to serological identification of serotypes has been restriction fragment analysis of the gene that encodes MOMP, *ompA*. This technique has been able to fingerprint serovars and intraserovar variants of *C. trachomatis.* Briefly, chlamydial species are first propagated using cell culture. A group of restriction enzymes are used to digest *ompA*; subsequent variations on the technique have used chlamydial genomic DNA or PCR amplified genes (i.e., *ompA* or 16S or 23S rRNA regions) [73]. The products are subsequently run on agarose or polyacrylamide gels where variation in the molecular weight size of the DNA fragments characterize the specific strains in comparison with control fragments representing each of the known strains. AFLP is a slight modification where chlamydial DNA is cleaved by a couple of restriction enzymes, then ligated to adapter oligonucleotides containing the same restriction enzymes. These products are amplified with fluorescently labeled primers specific for the adapter-specific primers. The fluorescent products are then separated on a polyacrylamide gel and analyzed for variations.

These molecular techniques have demonstrated a high level of agreement with serotyping. Additionally, PCR amplification of *omp2* and digestion with HinfI, RsaI, and TaqI has been able to differentiate the nine different *Chlamydiaceae* species [74]. In clinical applications, RFLP of *ompA* from nested-PCR for *C. trachomatis* serovar Ga revealed an association with symptomatic urethritis in male patients, whereas serovar Ia was found to be more frequently associated with asymptomatic infection [75]. RFLP has also been used to identify differences among polymorphic membrane proteins genes (*pmp*) E, H, and I as well as conserved *pmp*s for 15 different *C. trachomatis* serovars [76]. Furthermore, groupings

of serovars by ocular, non-invasive urogenital disease and invasive disease (serovars A-C, D-K, and LGV, respectively) were identified after one restriction enzyme (*CfoI*) digest of *pmp*H [76].

AFLP has been used to characterize and distinguish among *C. pneumoniae, C. psittaci, C. pecorum,* and *C. trachomatis* species [77], and for typing *C. psittaci* [78] and *C. pneumoniae* [79]. The use of one restriction enzyme, *Sal*I, on 23 different strains of *C. psittaci* distinguished the host origin of infection resulting in two avian, one feline and muskrat, and one ruminant group [80]. In contrast to *C. trachomatis* and *C. psittaci, C. pneumoniae* contains a high degree of intra-strain homology resulting in little to no variation with RFLP or AFLP [77].

Once a diverse segment is identified, genomic sequencing could identify polymorphic regions and specific mutations to aid epidemiologic studies. Additionally, it might assist research scientists in identifying microbial protein structures responsible for tissue and/or host specificity. RFLP is therefore a relatively cheap method to scan genome looking for genes of high diversity, although point mutations (i.e., SNPs) outside of restriction sites could be missed depending on whether there was any variation in the resulting molecular weight of the fragment that would migrate through the gel. Other disadvantages include the inability to detect mixed infections either with multiple serovars as in STIs or different species as in trachoma.

20.5.4 ompA Genotyping

MIF and serotyping highlighted the variance in MOMP epitopes, but scientists were interested in developing a molecular fingerprinting method that presumably would have greater resolution at the *ompA* gene level. The initial procedure used cell culture propagation to acquire *C. trachomatis* RNA, which was reverse transcribed into cDNA. γ-P^{32} labeled sequencing primers were subsequently hybridized to cDNA templates and analyzed on polyacrylamide sequencing gels. This genetic analysis of the four variable segments of *ompA* confirmed the serotyping segregation of *C. trachomatis* strains into the three serogroups [81]. Modification of the protocol with the use of PCR amplification has resulted in elucidating many strain types or subtypes within these serovars among both trachoma and STI samples [67, 82]. Additionally, this technique has identified *C. psittaci* and *C. pneumoniae* as new prevalent etiologic agents of trachoma [64]. Multiple new intra-serovar *ompA* variants have also been identified among a population of men having sex with men [83]. *ompA* sequencing revealed a significant level of intra- and interspecies recombination [84], which has evolutionary implications for the emergence of new strain types that may exploit new host niches.

C. pneumoniae has been differentiated into four different genotypes (A–D) based on mutations in *ompA* among clinical strains [85]. This genotyping technique has also highlighted possible zoonotic transmission where it has been used to identify genomic differences among isolates from koalas, frogs, horses, and boas [86].

Overall, with the use of PCR on small concentrations of bacterial genomic DNA and the availability of sequencing facilities, *ompA* genotyping has significantly expanded clinical and epidemiologic studies as well as shed light on *Chlamydia* evolution. Moreover, the approach has become economically feasible.

20.5.5 Microarrays

Rapid advancements in the biotechnology field have increased the number of genes that can be analyzed on microarrays and the number of samples that can be run at one time, making the arrays more economically feasible. However, there has been limited array development for chlamydial research, although the technology was first described over 20 years ago. Currently, microarrays have evolved to quantify expression levels of DNA, RNA, or protein. Basically, an array or chip typically made of glass, plastic, or silicon is labeled with thousands of microscopic spots containing probes specific for an oligonucleotide, gene, or protein. The techniques have evolved to include genome hybridization where a genome is hybridized to the array and compared to the genome of a closely related strain within the same species, although deletions, inversions, and recombinant regions are difficult if not impossible to detect. However, this technique has been able to identify polymorphic regions within the plasticity zone (PZ) among 15 *C. trachomatis* ocular and genital serovars [87]. These mutations were further characterized using PCR and sequence analysis to highlight multiple deletions within the cytotoxin loci of *C. trachomatis* [88]. More recently, research scientists have utilized this technology to characterize bioactivity associated with putative and known chlamydial proteins [89, 90] and identify immunodominant proteins that can be tested as candidates for vaccine development [91]. Microarrays have also been used to differentiate *Chlamydiaceae* species commonly found in animals [92]. With public access to microbial genomic sequences, bioinformatic comparative analysis assigns putative proteins into protein functional groups due to sequence homologies. Confirmation of these biological functions can be determined by overexpression in well-characterized microbial hosts, like *E. coli,* with subsequent microarray analysis.

Recent advances in technological readouts have improved the utilization of microarrays in the medical field, benefiting both the epidemiologist and clinician. Expensive optical scanning equipment initially discouraged clinical applications, but recently a semiconductor microchip was developed that enables electrochemical detection, making this technology more economically feasible [93]. It has been used to identify the etiologic agents of multiple upper respiratory tract infections through the development of probes against four bacterial pathogens, including *C. pneumoniae,* and nine viral pathogens. Similarly oligonucleotide microarrays have been successfully used to discriminate between etiologic agents of sexually transmitted diseases [94, 95]. Another study used 35 hybridization probes against VS2 and 4 of the *ompA* gene for *C. psittaci* to reconfirm the nine known genotypes of the organism and was able to identify 20 additional single nucleotide polymorphism (SNPs),

representing different *ompA* genotypes of the organism [96]. However, only 25 SNPs have been identified within *C. trachomatis* trachoma strains (A2497, B/HAR-36, C/TW-3, and A/HAR-13) [97] using a microarray-based comparative genome sequencing protocol originally described by Albert *et al.* [98], suggesting that comparative genomics is required to differentiate similar strains with high fidelity. Nonetheless, the versatility of microarrays have resulted in a wide range of applications from the lab bench to the bedside. The future application of protein microarrays to chlamydial biology will further aid our understanding of the pathogenicity of the diverse diseases caused by *Chlamydiaceae* species.

20.5.6 Multi-Locus Sequencing Typing

While *ompA* genotyping has provided a greater discrimination of strain types than other typing techniques, the fact that it cannot differentiate strains associated with different disease states suggested that other typing strategies were needed. Multi-locus sequence typing (MLST) schemes have been developed for over 30 human pathogens and have been used for monitoring and characterizing disease-causing lineages and outbreaks for a variety of pathogens associated with human diseases of significant morbidity and mortality [99, 100] such as *Vibrio cholerae* [101], *Staphylococcus aureus* [102], *Haemophilus influenzae* [103], and *Neisseria gonorrhoeae* [104], to name a few. The schemes use 6–8 housekeeping genes and PCR amplification and sequencing of approximately 500–700 base pairs of each gene for comparative genetic analyses. There are currently three different MLST schemes for *C. trachomatis* [105–107]. The first published scheme utilized genes that are under immune selection [105], which violates the premise of evaluating only genes that are considered essential to the housekeeping function of the organism. Sequence analyses using the second published scheme, which also included an MLST scheme for *C. pneumoniae*, have demonstrated and confirmed the taxonomy associated with the family *Chlamydiaceae* [106]. The third scheme has identified approximately three times as many intra-serovar variant strains within *C. trachomatis* compared to *ompA* genotyping in addition to identifying disease defining strains as well as strains that are recombinants of two or more serotypes [107]. In addition, the latter study identified SNPs that were significantly associated with invasive STDs (LGV strains), non-invasive prevalent STDs (strain types D, E, and F), and trachoma (strains A, B, Ba, and C).

Overall, this methodology has the benefit of using DNA directly from clinical samples and increasing the sensitivity for detecting new intra-serovar variants while retaining genus and species specificity. In theory, analysis of multiple loci from the same samples should also carry an increased confidence level in the results by reducing false positives. With the advent of new and improved high throughput sequencing capabilities in addition to online access to sequence analysis tools [100], MLST has become a feasible approach to detecting strain types for epidemiologic and basic research studies of trachoma and STD populations. However, MLST is still confined to specialized research laboratories because of the technical expertise required.

20.5.7 Genome Sequencing

The advent of modern sequencing technology has made this technology more economically feasible. To date, only 14 serotypes of *C. trachomatis* have been genome sequenced: A/HAR13, B/Jali20/OT, B/TZ1A828/OT, D/UW-3, E/11023, E/150, G/9301, G/9768, G/11222, G/11074, L_2/434/Bu, and L_2b/UCH-1/proctitis (available at GenBank: www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj&cmd=search&term=Chlamydia+trachomatis). A/HAR13, D/UW-3, and L_2/434/Bu are reference strains initially isolated in the 1950s–1960s that were adapted to cell culture and maintained in various labs since then. These reference strains most likely do not reflect the *C. trachomatis* bacteria responsible for disease in human populations today. Indeed, there is growing evidence for intra- and inter-species recombination that have implications for the emergence of more or less virulent *Chlamydiaceae* pathogens [84, 108–110]. Five human and one koala stain have now been genome sequenced for *C. pneumoniae* (available at GenBank). Two genome sequences are available for *C. psittaci* (available at GenBank). There are several strategies available for whole genome analysis of chlamydial strains. It is now possible to generate whole genome shotgun single read 454 GS-FLX Titanium instrument (FLX™) data. The FLX™ instrument is currently capable of sequencing ~400 Mb per run with an average read length of >400 nucleotides. The Illumina GAII system is an alternative for sequencing genomes to higher depth, but the short reads (2×100 nucleotides) are less useful for mapping recombinants and de novo sequencing. As the technology advances, we are approaching the under $1,000 per chlamydial genome mark with an average read redundancy of 20, making genome sequencing accessible for a wide variety of research purposes. Comparative and functional genomics will open up our understanding of the current diversity of chlamydial strain types for all three human *Chlamydiaceae* pathogens, including the nature of their evolution, the emergence of new strain types, the expansion within current and new anatomic niches, the extent of intra- and inter-species recombination and the mechanisms involved, and the mechanistic possibilities for animal *Chlamydiaceae* species to jump species to humans. Moreover, it may be possible to develop a gene transfer system for chlamydiae to explore disease pathogenesis and develop new drug targets and an effective vaccine.

20.6 Point-of-Care Chlamydia Tests

We have discussed different methodologies used to detect chlamydial infections among patient populations. Current diagnostics rely mostly on commercial NAATs, serology, or tissue culture that are all problematic. These tests vary in sensitivity and specificity with a much lower sensitivity for culture and a general lack of concurrence among the NAAT tests for sensitivity as discussed above. They are also expensive and require technical expertise, delaying the time to diagnosis by days or even weeks, especially in the case of batch processing of clinical samples or the need for culture. This is problematic in that some infected patients may be lost to follow up,

disease may progress, and transmission may increase. Also, the expense precludes rational screening, which would detect many of the asymptomatically infected men and women. As *C. trachomatis* infections increase (there are over 92 million cases that occur globally each year [111] and over one million annual cases in the United States alone [112]) *a more suitable and reliable* screening strategy is in demand. While there is a new *Chlamydia* rapid test, it is based on antigen detection and has an unacceptably low sensitivity of 84% [113]. If a rapid, cost-effective and sensitive diagnostic became available for *C. trachomatis*, there would be an incentive to widely screen for the organism thus (1) increasing early detection; (2) informing appropriate treatment; (3) reducing unnecessary antibiotic use; (4) providing proof-of-cure; (5) radically reducing the rates of infections in both symptomatic and asymptomatic individuals and complications in all age groups (including PID, infertility, ectopic pregnancy, chronic pelvic pain, suppurative lymphadenitis, hemorrhagic proctitis, rectal strictures, and infant pneumonitis); (6) substantially impacting the national and international cost for chlamydial STDs; and (7) providing a critical means for assessing the efficacy of new drugs and vaccines. These potential individual and societal benefits make a compelling argument for the development of a rapid, cost-effective, sensitive and specific diagnostic for *C. trachomatis*.

20.7 Future of Molecular Typing

While next generation genome sequencing is making fast inroads into producing accessible genomic data that will greatly inform detection and strain typing for *Chlamydiaceae* species, rapid point-of-care (POC) diagnostics and strain typing methodologies are necessary to appropriately screen and treat at-risk populations to not only decrease the prevalence of chlamydial diseases but to acquire epidemiologic data necessary to design appropriate preventive intervention strategies. Detection methodologies that use microfluidics bring together nanotechnology, PCR, and sequencing that can use microliter volumes of clinical samples and, theoretically, result in high specificity and sensitivity. In theory, microfluidics is the "wave" of the future where many of the molecular technologies discussed above are "assembled" into a single instrument for a rapid, higher resolution, portable and yet more affordable technique. Innovative studies in microfluidics have decreased contamination of DNA, reduced sample volume size, achieved sufficient DNA isolation from single cells, and performed whole genome amplification. Basically, some of the devices have incorporated a cell sorter, aiding in single cell isolation. Subsequent genomic amplification of this cell occurs within four unique chambers: template, alkaline cell lysis and DNA denaturation, neutralization, and finally the reaction chamber. Additional technological advances have incorporated a new DNA polymerase from the bacteriophage φ29 allowing for more efficient chromosomal amplification through multiple displacement amplification (MDA) of single cells [114]. With the continual emergence of antibiotic resistant strains of microbes, microfluidic systems have also been able to isolate microorganisms on a single cell basis to determine antibiotic sensitivity [115].

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Part V
Vector Borne Pathogens

Chapter 21
Borreliae

Dionysios Liveris, Klára Hanincová, and Ira Schwartz

21.1 Introduction

Borreliae are classified in the order Spirochaetales. All members of the genus *Borrelia* are highly motile, spiral-shaped organisms. The cells are comprised of a protoplasmic cylinder enclosed by an inner and outer membrane [1]. The periplasmic compartment contains a number of endoflagella which are responsible for both cellular motility and helical cell shape [2]. The outer membrane is devoid of lipopolysaccharides, but contains a large number of lipoproteins [3, 4]. Common features of members of this genus are (1) vertebrate host association, (2) transmission to new hosts by hematophagous arthropod vectors parasitized with borreliae, and (3) relatively low mol% G+C content (27–32%) in their DNA [1]. All borreliae cultured to date are microaerophilic, slow growing, and require complex culture media for propagation [1]. The genus *Borrelia* is divided into two major pathogenic groups—the Lyme disease (LD) borreliae and relapsing fever (RF) borreliae. The Lyme disease group of *Borrelia* consists of at least 14 different species and some members of this group are the agents of Lyme disease. The relapsing fever group includes more than 20 *Borrelia* species [5] that are agents of tick-borne (TBRF) or louse-borne (LBRF) relapsing fever (27–32%) [6]. LD borreliae in nature are maintained in enzootic cycles involving a variety of mammalian and avian hosts and hard ticks of the genus *Ixodes* as vectors. The primary bridging vectors to humans are *Ixodes scapularis and I. pacificus* in North America, *I. ricinus* in Europe, and *I. persulcatus* in Asia [7, 8]. Vectors of the RF *Borrelia* agents are soft-bodied ticks (family Argasidae), mainly of the genus *Ornithodoros*; the one notable exception is *Borrelia recurrentis,* which is transmitted by the human louse (*Pediculus humanus humanus*) [9].

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Many aspects of *Borrelia* spp. biology, epidemiology, and pathogenesis have been thoroughly covered in previous reviews [1, 9–11]. In this chapter, we focus on the different molecular methods employed in species identification, typing and subtyping of LD and RF borreliae. The contribution of these typing methods to taxonomy, epidemiology, and diagnostics will be discussed.

21.1.1 Molecular Typing of Borreliae

B. burgdorferi was identified as a new human pathogen in 1983 [12–14]. Since then, hundreds of isolates have been cultured worldwide from *Ixodes* ticks, small mammals and birds and from Lyme disease patients. Molecular characterization of these isolates has shown that they are genetically diverse [15–22]. Taxonomic classification of LD spirochetes has been revised in the last two decades based on information obtained from molecular typing methods with increasing discriminatory power [10, 23, 24]. Presently, 14 species comprise the cluster of genetically related isolates [10, 23, 25–27]. Five species (*B. burgdorferi*, *B. andersonii, B. bissettii, B. californiensis,* and *B. carolinensis*) have been detected in the United States and only *B. burgdorferi* has been associated with human disease. The remaining nine species (*B. afzelii, B. garinii, B. japonica, B. lusitaniae, B. sinica, B. spielmanii, B. tanukii, B. turdii,* and *B. valaisiana*) have been identified exclusively in Eurasia. In this latter group, only *B. afzelii and B. garinii* have been definitively associated with human Lyme disease, although limited human cases of infection with *B. bissettii*, *B. valaisiana,* and *B. spielmanii* have been reported [28–31]. Human infection by these *Borrelia* species usually results in a characteristic skin rash, erythema migrans (EM) [32, 33]. Dissemination of the spirochetes from the initial site of infection in skin can result in extracutaneous manifestations that may be dependent on the infecting species [15, 34]. *B. burgdorferi* infection more frequently causes Lyme arthritis, whereas infection with *B. garinii* and *B. afzelii* is more frequently associated with neuroborreliosis and a chronic skin condition (acrodermatitis chronica atrophicans), respectively.

Classification of RF borreliae has been traditionally based on geography, e.g., the Afro-tropical species of *B. duttonii* and *B. crocidurae* as Old-World TBRF and *B. hermsii* and *B. turicatae* found in the western and south-central US as New-World TBRF species [35]. The specific relationship between spirochetes and their arthropod vectors has been used as a means of speciation of RF borreliae [5, 36, 37]. Specifically, *B. hermsii* and *B. turicata* are exclusively transmitted by *O. hermsii* and *O. turicata* ticks [38]. Several new species of RF agents collectively named *B. miyamotoyi* sensu lato have been recently reported [9]. These include *B. miyamotoyi*, *B. miyamotoyi*-like, and *B. lonestari* transmitted by the hard ticks *I. persulcatus*, *I. scapularis,* and *Amblyomma americanum*, respectively [39–41]. The disease potential of these species is currently unknown. *B. miyamotoyi* has not been isolated from humans and only a single case of human infection with *B. lonestari* has been reported [42].

21.1.2 Phenotypic Typing

Conventional bacterial phenotyping approaches such as biotyping, antibiotic susceptibility profiling, and bacteriophage typing cannot be applied to the genus *Borrelia* due to the extreme fastidiousness of the organisms and the inability to form confluent lawns on solid media. Although a phage has been reported for *B. burgdorferi* [43–45], its utility for strain typing is unknown. Typing of *Borrelia* by fatty acid content and total protein profiling by SDS-PAGE are unreliable since both methods require defined growth media and specific metabolic states of the cells being typed [10]. Multi-locus enzyme electrophoresis (MLEE) typing, which involves comparison of the mobility of metabolic enzymes on gel electrophoresis, has been employed in a limited number of studies, but its utility for *B. burgdorferi* is limited because it is labor intensive and requires large amounts of cultured organism [10, 46, 47]. Serotyping of LD spirochetes based on two outer surface proteins, outer surface protein A (OspA) and outer surface protein C (OspC), has provided some important insights into vaccine development and clinical outcome, but this approach has been supplanted by genetic typing methods [48–51].

Phenotyping of RF borreliae has been hampered by the inability to cultivate many of these species and is not in common use. Biotyping has been largely based on vector specificity and geographic location. Biological assays, such as guinea pig resistance to *B. duttonii* [52], primate susceptibility to *B. recurrentis* [53], and bird susceptibility to *B. anserina* [54], have also been employed. In addition, for cultivable species, variations in sugar fermentation [55] and in structural traits, such as the number of flagella [56], have been reported. Serotyping of RF borreliae has been reported, but its applicability for typing is questionable since these species undergo continuous antigenic variation in mammals [35].

21.1.3 Genetic Typing

A variety of genetic typing methods, targeting both chromosomal and plasmid loci, have been applied to *Borrelia* species. These include DNA–DNA relatedness analysis, rRNA ribotyping, pulse-field gel electrophoresis (PFGE), plasmid fingerprinting, species-specific PCR, PCR-based restriction fragment length polymorphism (PCR-RFLP) analysis, single-strand conformational polymorphism (SSCP) typing, DNA sequence analysis of species-specific genes, variable-number tandem repeat (VNTR) analysis, and multi-locus sequence typing (MLST). In general, these approaches can be divided into those that require purified DNA (DNA–DNA hybridization, ribotyping, PFGE, plasmid fingerprinting) and those that can be accomplished by PCR with less purified material. The former methods are cumbersome and require in vitro cultivation of the spirochetes. Information gathered from these analyses is often used for bacterial species identification and taxonomy. In contrast, PCR-based typing schemes are relatively simple, rapid, and can be performed directly on large numbers of environmental or patient samples without the necessity of prior

cultivation. Results obtained from these latter analyses can be employed for both species/strain differentiation and elucidation of population dynamics of *Borrelia* species in nature. Furthermore, the potential problem of clonal selection of isolates by in vitro cultivation is avoided [21, 57]. In the following sections, each of these methods and their applications are discussed.

It should be noted, of course, that the ultimate typing approach is complete genome sequencing. The genome of *B. burgdorferi* strain B31 was among the first bacterial genomes to be completely sequenced. This revealed a unique genetic structure among prokaryotes, as the complete genome was comprised of a single, large linear molecule (referred to as the chromosome) of 910 kb and a collection of 12 linear and 9 circular plasmids with a combined size of approximately 600 kb [58, 59]. Although the genomic sequences of other *Borrelia* species are deposited in GenBank, that for strain B31 remains the only *fully* sequenced genome; the remaining genome sequences lack information on varying numbers of plasmids. The genomic features described for the *B. burgdorferi* B31 genome are also characteristic of other LD and RF *Borrelia* species sequenced to date in that they are comprised of a large linear chromosome and multiple linear and circular plasmids [60, 61]. The complete genomes of the RF borreliae, *B. reccurentis, and B. duttoni* have been reported [62] and those of *B. hermsii* and *B. turicatae* have been deposited in the NCBI genome database.

21.1.3.1 Genetic Typing Methods Employing Purified Genomic DNA

DNA–DNA Hybridization

The reference method for measurement of DNA relatedness between two organisms is DNA–DNA hybridization. This approach was employed for speciation of LD borreliae [63, 64]. The strength of this method is based on the fact that classification relies on comparison of total genomic sequences for the organisms under investigation. A DNA relatedness of 70% is the cut-off for defining members of a given species [65]. DNA homology among LD *Borrelia* species ranges from 48 to 70%, whereas that between LD and RF borreliae range from 30 to 44% [14, 63].

For North American RF spirochetes, *B. hermsii* shows 86 and 77% homology to *B. turicatae* and *B. parkeri*, respectively; DNA relatedness between *B. hermsii* and other RF agents is much lower (17–63%) [1]. A confounding factor is the variable plasmid content observed among *Borrelia* species and isolates. DNA–DNA hybridization of *B. turicatae* and *B. parkeri* suggests that they are members of a single species but the absence of circular plasmids in the latter and transmission of each species by a unique vector tick species suggests that they are distinct species [38].

Plasmid Typing

All members of the genus *Borrelia* contain multiple linear and circular plasmids. The number of plasmids and their molecular size vary among isolates of LD borre-

liae [66–69]. This variation has been the basis of plasmid fingerprinting as a typing method. For example, among 40 LD isolates from different sources and geographical locations, plasmid fingerprinting data correlated with *Borrelia* species designations by other typing methods, but no species-specific plasmids were identified [70]. The reported loss of both linear and circular plasmids during in vitro propagation and the possibility of plasmid recombination limit the usefulness of this method for LD agent typing [71–73]. This may be less of a problem for RF *Borrelia* species [74]. However, no comprehensive plasmid fingerprinting studies have been yet described for RF borreliae. The reported absence of circular plasmids from the genomes of *B. parkeri*, *B. anserina,* and *B. recurrentis* may be useful for differentiating these RF species from other RF borreliae [38].

Ribotyping

Ribosomal RNA genes are highly conserved and are universally present in bacteria. This facilitates the use of *E. coli*-based probes for identification of restriction fragments containing rRNA genes for many bacteria. The technique involves digestion of total genomic DNA with one or more restriction enzymes, electrophoretic separation of restriction fragments and Southern blotting with a probe for conserved regions of rRNA [75]. Individual species of *B. burgdorferi*, *B. garinii*, and *B. afzelii* can be identified by specific *Hin*dIII DNA fragments [15]. A study of 51 LD isolates demonstrated that all 18 *B. burgdorferi* strains belonged to a single ribotype, while 23 *B. garinii* and 10 *B. afzelii* isolates were distributed into nine and three ribotypes, respectively [76]. The method is relatively simple and highly reproducible, but has been superseded by PCR-based methods targeting rRNA genes (see below).

Pulsed-Field Gel Electrophoresis (PFGE)

A second typing method based on restriction enzyme digestion of total genomic DNA is pulsed-field gel electrophoresis. This approach employs restriction enzymes whose recognition sequences rarely appear in the *Borrelia* genome such that digestion of total genomic DNA produces a relatively small number of large restriction fragments. *Mlu*I has been the most useful enzyme for PFGE analysis of LD spirochetes. Most species can be identified by characteristic species-specific digestion fragments (e.g., 135 kb for *B. burgdorferi*, 220 kb and 80 kb for *B. garinii,* and 460, 320, and 90 kb for *B. afzelii*) [77, 78]. Strain differentiation within a species can also be accomplished by PFGE. Among 20 *B. burgdorferi* strains there were 10 *Mlu* I types and among 24 *B. garinii* strains there were 4 *Mlu* I types; interestingly, no variation was observed among 20 *B. afzelii* isolates [77]. *Mlu*I-based PFGE analysis of 186 North American *B. burgdorferi* isolates revealed 19 different patterns, although just a few of these predominated [18]. Six different PFGE types were identified in 48 *B. burgdorferi* clinical isolates from early Lyme disease patients [79]. In general, there is excellent correspondence between PFGE and other typing

methods [18, 79]. PFGE has also been beneficial for constructing physical maps of the *B. burgdorferi* chromosome [80, 81]. PFGE analysis remains a valuable tool for *B. burgdorferi* typing, but a drawback is the requirement for culture and large amounts of purified DNA.

21.1.3.2 PCR-Based Typing Methods

DNA Sequencing of PCR-Amplified Loci

Genetic typing methods involving PCR amplification of various genomic targets of LD borreliae are relatively simple, fast, and discriminative at the species and, in some instances, at the isolate level [17, 24, 82–84]. Analysis of PCR-amplified 16S rRNA products by either RFLP analysis [85] or by direct DNA sequencing results in species-level differentiation among LD borreliae [86]. DNA sequence homology among *B. burgdorferi*, *B. garinii*, *B. afzelii*, *B. lusitaniae,* and *B. valaisiana* at the *rrs* (16S rRNA) locus varies from 95.3 to 99.6% [86]. The GenBank database contains over 100 *rrs* sequences for LD *Borrelia* species. While these sequences are useful for species identification, they are less suitable in discriminating between strains of the same species. Numerous other genetic loci have been employed to identify LD *Borrelia* species and to investigate the taxonomic and evolutionary relationships among these spirochetes. These include the genes encoding flagellin (*flaB*) [87–89], outer surface protein A (*ospA*) [18, 87, 90], outer surface protein C (*ospC*) [88, 91], P66 (*p66*) [84, 92], histone-like protein (*hbb*) [93], P39 (*bmpA*) [94], and heat-shock proteins (*hsp60* and *hsp70*) [87]. Dykhuizen and co-workers have characterized over 20 different *ospC* genotypes in North America and Europe and *ospC* sequence analysis has become a primary tool for *B. burgdorferi* genotyping. Several studies have correlated *ospC* genotype with disseminated infection in LD patients [95–102]. Despite the substantial utility of *ospC* for genotyping, however, evidence of *osp*C intragenic recombination and lateral transfer between strains makes *ospC* unsuitable for phylogenetic studies [103–105].

DNA sequence variation at the *fla*B locus allow for discrimination between LD and RF *Borrelia* species [41, 89]. *glpQ,* a gene encoding glycerophosphodiester phosphokinase, is found in all RF borreliae, but not in LD species and thus can be employed for identification of RF agents [106]. Sequence variation in *glpQ* was used to differentiate between *B. lonestari* and *B. miyamotoi* [107]. Sequence variation at the *vtp* locus of *B. hermsii* (an ortholog of *ospC*) has been described; however, the utility of this target for RF species typing is unknown [108].

Ribosomal RNA Spacer RFLP Analysis

Genome analyses have shown that all LD *Borrelia* species possess a unique rRNA gene organization that is different from that of other prokaryotes, including RF *Borrelia* species [109]. The region consists of a single 16S rRNA gene (*rrs*) followed

by a large intergenic spacer (IGS); 3.2 kb in *B. burgdorferi* and 5.0 kb in *B. garinii* and *B. afzelii*. This is followed by a tandem repeat of 23S rRNA-5S rRNA genes (*rrlA-rrfA—rrlB-rrfB*); the tandem copies are separated by a short spacer of 225–266 bp [58, 110–112]. It is assumed that the rRNA IGS accumulate higher levels of sequence variation between related species than do coding regions because IGS regions do not encode functional gene products.

rRNA spacer restriction fragment length polymorphism (PCR-RFLP) typing has been extensively applied to study LD *Borrelia* species. PCR amplification of either *rrs-rrlA* or *rrfA-rrlB* intergenic spacers, followed by RFLP analysis with *Mse* I, allowed differentiation of LD species [16, 17, 113–116]. Postic et al. employed *Mse*I digestion of PCR amplified *rrfA-rrlB* IGS for differentiation of eight LD *Borrelia* species [16]. A modification of this typing method based on reverse line blotting of the *rrfA-rrlB* PCR product followed by hybridization with species-specific DNA probes enabled investigators to directly assess presence of different LD species in ticks [113]. Use of single strand conformational polymorphism (SSCP) in conjunction with PCR amplification of the *rrfA-rrlB* IGS has also been reported [117]. This facilitated identification of a new variant isolate of *B. burgdorferi* previously undetectable with RFLP analysis. The relatively small size of the *rrf*A–*rrl*B IGS (225–266 bp) limits the amount of information that can be obtained by RFLP analysis for typing of individual isolates.

PCR amplification of the 941 proximal base pairs of the *rrs-rrlA* spacer, encompassing the region immediately downstream of *rrs* and terminating at the conserved *ileT* locus, followed by RFLP analysis with either *Mse*I and *Hinf* I restriction enzymes, was able to distinguish LD borreliae at the species level and, more importantly, *B. burgdorferi* at the strain level [17, 21, 115, 116]. Isolates with different RFLP profiles were designated as ribosomal spacer types RST1, RST2, and RST3. Studies using this typing method with uncultivated *B. burgdorferi* in human tissues and field-collected ticks have shown that both LD patients and ticks may be simultaneously infected with one or more distinct genotypes of *B. burgdorferi* [21, 118]. Numerous studies have demonstrated that RST genotype correlates with pathogenic potential in humans and mice [17, 21, 102, 115, 116, 119–122]. A recent study of more than 400 *B. burgdorferi* clinical isolates showed that a combination of RST and *ospC* genotyping permitted the identification of *B. burgdorferi* genotypes that pose the greatest risk of hematogenous dissemination in humans [102].

Bunikis et al. modified this method by direct sequence analysis of the IGS PCR products [84]. They concluded that polymorphism in the first 250 nucleotides of the *rrs-rrlA* IGS was sufficient to discriminate between genotypes; 68 *B. burgdorferi* isolates could be classified into 10 distinct IGS genotypes and the 107 isolates of *B. afzelii* tested were classified into 11 IGS types [84]. By sequencing the entire IGS, Hanincova et al. were able to identify 16 IGS alleles among 127 *B. burgdorferi* clinical isolates [123]. The results of these studies also demonstrated linkage disequilibrium between the *rrs-rrlA* IGS and *ospC* loci [84, 102, 123]. A correlation between RFLP-generated RST types and sequence-generated IGS types concluded that IGS typing of the *rrs-rrlA* partial spacer was sufficient for strain typing of *B. burgdorferi* [84].

IGS typing has also been applied to New World and African RF *Borrelia* species [124, 125]. This facilitated differentiation of *B. hermsii*, *B. turicatae*, *B. miyamotoi,* and *B. lonestari* in North America [124]. *B. crocidurae* could be distinguished from *B. recurrentis* and *B. duttonii*, but this approach did not permit differentiation between the latter two African species [125].

Real-Time PCR

Differentiation of LD *Borrelia* species using real-time quantitative PCR targeting *p66* or *recA*, followed by melting curve analysis, has been described [126]. A simplified method of speciation and quantitation of *B. burgdorferi*, *B. garinii,* and *B. afzelii* has been developed and successfully applied to *I. ricinus* ticks in Germany. This typing method involved a single-run real-time PCR reaction targeting *ospA* and melting curve analysis of the amplified products to distinguish the *Borrelia* species [127]. Of the 1,055 *I. ricinus* ticks tested, 35% were infected; 53% with *B. afzelii*, 18% with *B. garinii* and 11% with *B. burgdorferi*, 0.8% could not be identified and 18% with mixed infections [127]. This approach has diagnostic value, but as yet has not been applied to strain typing.

Multi-Locus Sequence Typing

Differential distribution of 10 variable-number tandem repeat (VNTR) loci was utilized for both species and strain identification in a group of 41 globally diverse LD isolates [128]. This analysis was able to identify *B. burgdorferi*, *B. garinii,* and *B. afzelii*, and to discriminate between strains of *B. burgdorferi* [128]. However, the inability to detect VNTR loci in all *Borrelia* samples is problematic.

Multi-locus sequence typing (MLST) was proposed in 1998 as a highly discriminatory technique that generates accurate data for epidemiological, evolutionary, and population studies of bacterial pathogens [129]. This technique uses a concept similar to multi-locus enzyme electrophoresis, but instead of electrophoretic mobility of metabolic enzymes, the alleles at each locus are defined by nucleotide sequence. A number of studies employed a combination of DNA sequence analyses at multiple genetic loci to characterize the genus *Borrelia* and to elucidate its population structure [23, 26, 84, 130, 131]. These multi-locus sequence analysis methods, however, differ from classic MLST since the analyzed loci included not only housekeeping genes, but other non-coding and plasmid-borne polymorphic loci. In contrast, classic MLST schemes use only housekeeping genes that slowly diversify by random accumulation of nearly neutral mutations and retain signatures of longer term evolution. To ensure highly discriminatory power of relatively uniform housekeeping genes, the combined sequences of multiple housekeeping gene loci are analyzed [132].

An MLST scheme for *B. burgdorferi* has been described that is based on a set of eight different housekeeping loci. The method was employed to evaluate 64 *B. burgdorferi* cultured isolates from North America and Europe [24]. Results indicated that

the North American and European populations of *B. burgdorferi* are genetically distinct and further suggest that *B. burgdorferi* may likely have originated in Europe and not in North America as has been previously thought [133]. In addition, it was demonstrated that the previously used genetic markers of *B. burgdorferi* such as the *rrs-rrl*A intergenic spacer and the *ospC* locus evolve differently, as compared to the eight housekeeping genes [24]. In a separate study, the MLST scheme was applied to a phylogeographical study of 16 specimens of *B. lusitaniae* isolated from ticks in two climatically different regions of Portugal [134]. MLST analysis was able to demonstrate that the *B. lusitaniae* populations from these two regions constituted genetically distinct subpopulations, which appeared monophyletic based on *ospC* and *ospA* phylogenetic analyses [134]. More recently, this MLST approach has been employed to demonstrate that the distribution of *B. burgdorferi* genotypes in ticks varies between the Northeastern and Midwestern United States [135], to study the phylogeography of *B. burgdorferi* in the United States [136] and to define a new *Borrelia* species [137]. Based on these recent studies, it is reasonable to expect that MLST will gain wider use for typing and phylogenetic analyses of *Borrelia* species.

21.2 Conclusion

Application of any typing method to borreliae depends on the objectives of the particular study, the level of resolution desired (species vs. strain), and the laboratory conditions and technical expertise available. The most specific information is provided by complete genome sequencing and DNA–DNA hybridization, but these methods require cultivation of the species of interest. Similarly, PFGE has been very useful for strain typing, but relatively large amounts of DNA are required. Since most *Borrelia* species are difficult to cultivate and grow very slowly (especially newly isolated species), typing methods involving PCR amplification are currently most commonly used. Among these approaches, sequencing of specific genes following PCR amplification and PCR-RFLP or rRNA spacer regions has been most widely used. The recently developed MLST method is likely to become the method of choice in the future.

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Chapter 22
Erysipelothrix

Yumiko Imada

22.1 Introduction

Erysipelothrix rhusiopathiae gives considerable economic losses to swine industry worldwide by sudden infectious deaths from acute septicemia and by total or partial condemnation of carcasses at meat inspection from subacute septicemia or chronic arthritis and endocarditis [7]. Although the control is done by vaccination, the cost of vaccination is also a loss to the swine industry. In modern pig farms, the most important sources of infection are carrier pigs and diseased pigs. Their urine, feces, saliva, etc., contain the bacterium and contaminate feed, water, litter, soil, etc. Pigs are infected by ingesting them, and become carriers or develop disease according to the virulence of the strain and their immune status. The control of the disease requires vaccination, stress-free good herd management, and removal of carrier pigs.

The contaminated pig manure may cause another infection in sheep and turkeys. In lambs, it causes polyarthritis. In turkeys, *E. rhuiopathiae* causes septicemic deaths, infertility of male, and marketing losses [9]. Turkeys are infected through cannibalism, fighting, and artificial insemination. In turkeys, control is done by immunization with killed or live vaccine.

Human infection usually manifests local skin lesion known as erysipeloid, occasionally develops arthritis or lymphadenitis, and rarely serious systemic complication with septicemia and endocarditis [8, 13]. The most important sources of human infection are contaminated fishes, crustaceans, and infected animals. Contrary to pigs, human infection is initiated by an injury to the skin with infective material and is closely related to their professions, e.g., fisherman, cook, veterinarian, butcher, and housewives. In case of endocarditis, the mortality is very high and sometimes it is misdiagnosed and mistreated. In human infection, the information of serovar is

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usually deficient and the epidemiological significance of serotyping is questionable [8], because human infection is sporadic, has very wide variety of sources, and is not transmitted from human to human.

On the other hand, in swine erysipelas, the epidemiological study of the causative strains is very important for the control. Although serological typing system has been used widely, it is insufficient for this purpose because most of the isolates from acute infectious septicemia belong to serovar 1a and most of the isolates from subacute and chronic infections belong to serovars 1a, 1b, and 2 [14–17]. So, the development of other typing methods is required for more precise epidemiological study of swine erysipelas. It is also important to discriminate live vaccine strain from virulent strain of serovar 1a. Molecular typing seems to be an important tool for these purposes. In this chapter, most of the pages are used for the description of genotyping, although protein pattern typing and enzyme pattern typing may also be molecular typing.

22.1.1 Phenotyping

22.1.1.1 Serotyping

For the epidemiological study of *Erysipelothrix*, serotyping by double agar-gel diffusion precipitation test has been used for long time. It is done by using heat extracted polysaccharide antigen and rabbit antiserum against formalin-killed bacterial cells [18]. At present, *Erysipelothrix* strains are classified into serovars 1a, 1b, 2a, 2b, and 3–26 [19]. Strains having no type-specific antigen are classified as group N. Most of the isolates from swine erysipelas belong to serovars 1 and 2. To the contrary about half of the isolates from healthy cattle, chicken and environment were untypable and most of the remaining isolates belong to serovars other than 1 and 2 [18, 20, 21]. Although Takahashi et al. [4] showed the relationship between the species and serovars, it is revealed that they do not necessarily correlate [6, 21–23].

22.1.1.2 Sodium Dodecyl Sulphate Polyacrylamide-Gel Electrophoresis

Tamura et al. [24] showed the possibility of species identification by sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) using 14 field isolates of *E. rhusiopathiae* and *E. tonsillarum* and reference strains of serovars 1–23. All strains were formerly identified by DNA-DNA hybridization. Although most of them showed species-specific patterns, three reference strains (one strain of *E. rhusiopathiae* and two strains of *E. tonsillarum*) showed protein patterns different from their species.

Bernáth et al. [25] applied SDS-PAGE and autoradiography to L-35S methionine labeled proteins of 12 *E. rhusiopathiae* strains and concluded that it is useful

for the identification of strains, because all strains differed from each other in their protein patterns.

22.1.1.3 Multilocus Enzyme Electrophoresis

Chooromoney et al. [26] examined the genetic diversity of 74 strains of *E. rhusiopathiae* of old classification isolated from a variety of animals and 22 reference strains of serovars by multilocus enzyme electrophoresis. They identified 50 electrophoretic types and two genetic clusters, cluster A for *E. tonsillarum* and cluster B for *E. rhusiopathiae*. However, by this method, two reference strains formerly identified as *E. tonsillarum* and *E.* sp.-1 by DNA–DNA hybridization [4] were classified as cluster B. In this study, the species of 74 isolates were not confirmed by DNA–DNA hybridization.

22.1.2 Genotyping

22.1.2.1 Plasmid Profiling

Noguchi et al. [27] detected plasmids in 7 out of 43 strains of *E. rhusiopathiae* of old classification isolated from pigs with chronic swine erysipelas. They prepared plasmid DNA by cesium chloride density gradient centrifugation. Because all strains showed different plasmid profiles, they suggested the possibility of using the profiles as an epidemiological marker. Eamens, Forbes, and Djordjevic [28] detected plasmids in 23 of 77 strains isolated from swine erysipelas by separating whole cell DNA by 1% agarose gel electrophoresis. Twenty-two of them belonged to serovar 2 and one belonged to serovar 1b. Plasmid sizes were 1.0, 1.2, 1.8, and 2.2 kb and they showed five profiles.

22.1.2.2 DNA–DNA Hybridization

Genomic DNA–DNA hybridization is the base of the bacterial taxonomy. By this method, Takahashi et al. [4] determined DNA relatedness among 23 serovar reference strains of *E. rhusiopathiae* of old classification and seven *E. tonsillarum*, and they revealed that genus *Erysipelothrix* comprised at least four species, *E. rhusiopathiae*, *E. tonsillarum*, and two other possible new species, referred to as *E.* sp.-1 and *E.* sp.-2 by Takeshi et al. [5]. Verbarg et al. [3] proposed other new species *E. inopinata* by this method. Takahashi et al. [6] identified 93 strains of *Erysipelothrix* isolated from a wide variety of sources by DNA–DNA hybridization and found one more new species, *E.* sp.-3. It remains unknown whether *E.* sp.-1 is *E. inopinata* or not because of the lack of DNA homology data, although the 16S rDNA nucleotide sequence of *E.* sp.-1 (GenBank accession; AB055907) and *E. inopinata* coincided.

22.1.2.3 Polymerase Chain Reaction

Genus Detection and Identification by PCR

Makino et al. [29] reported polymerase chain reaction (PCR) for the identification of genus *Erysipelothrix*. It utilized 16S rDNA sequence and produced 407 bp PCR products. When the template DNA was prepared by lysozyme *N*-acetylmuramidase treatment and phenol-chloroform extraction, the sensitivity was very high as less than 20 colony forming units per spleen from mice. They concluded that all *Erysipelothrix* strains detected by this PCR system in diseased pigs are *E. rhusiopathiae* because only *E. rhusiopathiae* is virulent for pigs. Although the 5′ region of the MO101 primer sequence is not correct, at present this method is widely used because of the high sensitivity and specificity and the ability to detect all members of genus *Erysipelothrix*.

Fidalgo, Wang, and Riley [30] modified the PCR by Makino et al. [29] to nested PCR to increase the sensitivity and applied it to 52 seafoods. The isolates were identified by PCR reported by Shimoji et al. [31]. Most of fish samples were positive by both culture and PCR and 14/15 isolates were *E. rhusiopathiae*. To the contrary, most of crustaceans samples were positive only by PCR. Yamazaki [32] developed a multiplex PCR using 16S rDNA and *E. rhusiopathiae* chromosomal DNA sequence reported by Shimoji et al. [31]. Although this method detected genus *Erysipelothrix* and *E. rhusiopathiae* as 719 bp and 2,210 bp bands respectively, further study is necessary because only type strains of *E. rhusiopathiae* and *E. tonsillarum* and five *E. rhusiopathiae* isolates were used in this study.

Genus Detection and Identification by Real-Time PCR

Akase et al. [33] applied the real-time PCR for the direct detection of *Erysipelothrix* from arthritic fluids of pigs using the 16S rDNA target reported by Makino et al. [29]. In this study, they compared commercial DNA extraction kits and got highest sensitivity by QIAamp DNA Mini Kit (Qiagen). *Erysipelothrix* was detected from 27/31 isolation positive fluids and from 6/48 isolation negative fluids. These results were explained by the difficulty of DNA recovery from viscous arthritic fluids and also by the characteristics of PCR which can detect dead or viable not culturable bacteria as well as live bacteria.

Species Identification by PCR

Shimoji et al. [31] reported *E. rhusiopathiae* specific PCR by the use of capsule formation genes. Because the sensitivity of this PCR is not so high, it is better to be applied to enrichment culture or isolates. Takeshi et al. [5] reported four *Erysipelothrix* species-specific PCRs using sequences of 23S rDNA and the noncoding region downstream of 5S rDNA. These methods are not a multiplex PCR and should be

applied to strains already identified as genus *Erysipelothrix* by biochemical test or genus-specific PCR by Makino et al. [29], because the specificity of this method is not so high. Hassanein et al. [21] applied these four species-specific PCRs to 79 isolates from the tonsil of healthy cattle and compared the results with those by serotyping. Thirty-six isolates were untypable and identified as *E.* sp.-1. Among 43 remaining isolates, only 19 isolates showed correlation between serovar and species as shown by Takahashi et al. [4]. We examined the species of 200 strains of serovars 1a, 1b, 2b, and others (50 strains respectively) isolated from swine erysipelas by species-specific PCR reported by Takeshi et al. [5]. All strains of serovars 1a, 1b, and 2 belonged to *E. rhusiopathiae* except one strain of serovar 2b belonged to *E.* sp.-2. However, 50 strains of other serovars contained a variety of species, one *E. tonsillarum* (serovar 16), two *E.* sp.-1 (serovars 11 and 12), and one possible new species (serovar 3). Among 1,962 isolates from swine erysipelas, only these 50 strains belonged to serovars other than 1 and 2. These results showed almost all isolates from swine erysipelas belong to *E. rhusiopathiae* (Imada, unpublished data).

Strain Discrimination by PCR-Restriction Fragment Length Polymorphism (RFLP)

Eamens, Forbes, and Djordjevic [28] applied PCR-RFLP to 74 strains of serovars 1a, 1b, 1b×21, and 2 isolated from swine erysipelas to examine whether the vaccine breakdowns occurred in Australia between 1995 and 1998 were caused by specific virulent strain or not. They amplified 630 bp fragment of surface protective antigen gene (*spaA*) [34] and digested the products with *Rsa*I. However, they could not find any difference between strains isolated from farms with and without vaccine break down, because by this method all strains showed identical fragments of 248, 82, and 60 bp.

22.1.2.4 Randomly Amplified Polymorphic DNA Analysis

Species Identification by RAPD Analysis

Okatani, Hayashidani, and Takahashi [23] examined 18 primers and described species-specific randomly amplified polymorphic DNA (RAPD). With primer NK51 reference strains of serovar 1–23 and type N and 56 isolates from a variety of source were classified into four groups, *E. rhusiopathiae*, *E. tonsillarum*, *E.* sp.-1, and *E.* sp.-2 respectively. The RAPD results of reference strains coincided with those of DNA–DNA hybridization and the results of isolate coincided with those of *E. rhusiopathiae* species–specific PCR reported by Shimoji et al. [31]. In this study they found that serovars are not necessarily correlated to the species. Contrary to ordinary PCR, in RAPD-PCR DNA should be prepared from pure culture and also the amount of template DNA in the reaction mixture should be accurate. This RAPD requires much amount of template DNA as 500 ng per reaction.

Strain Discrimination by RAPD

Okatani, Hayashidani, and Takahashi [23] also showed the possibility of strain discrimination by RAPD by changing the species-specific primer NK51 to another primer NK6. With primer NK6 reference strains of serovars 1–23 and type N and 56 isolates from a variety of sources were classified into 25 genotypes, a to h corresponded for *E. rhusiopathiae*, j to l for *E. tonsillarum*, m for *E.* sp.-1, and n for *E.* sp.-2. Makino et al. [35] tried to discriminate Japanese live vaccine strains from virulent strains by RAPD using primers AP46 and AP47; however, by this method they could not prove the relationship between RAPD type and acriflavin resistance, one of the markers of Japanese live vaccine strain, Koganei 65-0.15. Imada et al. [17] examined ten primers and successfully discriminated Japanese live vaccine strains from virulent field strains of serovar 1a by the use of D9355 primer reported by Akopyanz et al. [36]. By this method, 381 strains of serotype 1a isolated from swine erysipelas were divided into four RAPD types, 1, 1-1, 1-2, and 1-6. RAPD type 1-2 correlated well to the acriflavin resistance and low pathogenicity in mice which were important markers of Koganei 65-0.15. Acriflavin resistance were found in 92% of 266 strains of RAPD type 1-2, while only in 1.7% of 115 strains of other RAPD types. The amount of template DNA for this RAPD is 20 ng or 2 μL of InstaGene Matrix (BioRad) template per reaction. Unfortunately D9355 prime is effective only for Japanese live vaccine strain and not for US strain.

22.1.2.5 PFGE

Okatani et al. [37] first applied PFGE to *Erysipelothrix* spp. and found *Sma*I was the most reliable enzyme. Seventy strains showed 63 distinct PFGE patterns with *Sma*I. They concluded PFGE might be more sensitive than RAPD with primer NK6 [23] and ribotyping with *Eco*RI [22]. Opriessnig et al. [38] modified the method to be completed within 2 days and applied it to 90 erysipelas isolates to investigate the marked increase of acute swine erysipelas in US in 2001. Most of the strains isolated in 2001 belonged to serovar 1a and showed the same genetic pattern 1A (I) apparently different from live vaccine strains. In this paper, the serovar 1a of live vaccine strains was mistook as serovar 1b.

We applied PFGE to 43 live vaccine strains and 55 virulent strains of serovar 1a isolated from swine erysipelas. Live vaccine strains showed eight patterns similar to Koganei 65-0.15 and 28/43 strains showed identical pattern to Koganei 65-0.15. To the contrary, virulent strains showed eight patterns which were different from vaccine strains (Imada, unpublished data).

22.1.2.6 Ribotyping

Ahrné et al. [22] classified reference strains of serovars 1–26 and field strains into nine ribopatterns using *Eco*RI. The classification correlated well to the species.

They first found serovar did not always correlate to the species although Takahashi et al. [4] had shown their relationship. Okatani et al. [39] applied automated ribotyping system to the reference strains of serovars 1–23 and field strains using *Eco*RI, and classified them into 27 ribogroups. They concluded it is more sensitive than traditional ribotyping and RAPD. Imada et al. [17] evaluated the ribotyping method using *Hind*III for the discrimination of live vaccine strains from virulent strains of serovar 1a. Totally 381 strains were divided into 12 ribotypes. Although the frequency distributions of the two groups were different, the separation of them was not clear-cut.

22.1.2.7 Restriction Fragment Length Polymorphic DNA Analysis (RFLP)

RFLP Without Hybridization

Eamens, Forbes, and Djordjevic [28] applied RFLP to 74 strains of serovars 1a, 1b, 1b×21 and 2, 44 strains from vaccine breakdown herds, 20 strains from nonvaccine failure, and ten reference strains, to examine whether the vaccine breakdowns in Australia between 1995 and 1998 were caused by specific virulent strain or not. In this experiment, chromosomal DNA was digested by *Rsa*I and analyzed by 3.5% polyacrylamide gel electrophoresis and silver staining of the gels. By this method, strains of serovars 1a, 1b, and 2 were classified into 10, 12, and 8 RFLP types, respectively. Because there was no geographic predominance of single serovar and 44 strains isolated from farms with vaccine breakdown showed ten *Rsa*I profiles, they concluded no single new clonal lineage of *E. rhusiopathiae* was responsible for vaccine failure.

RFLP Based on Hybridization

Imada et al. [17] found that 253 bp RAPD band (GenBank AB159679) specific to Japanese live vaccine strain is multicopy and used it as a probe of RFLP typing of serovar 1a strains. The sequence was identical to 5′ upstream region of putative transposase gene reported by Makino, Katsuta, and Shirahata [40]. By this RFLP, 29 live vaccine strains were classified into the same RFLP type A and 21 virulent strains were into other types B and C.

We further examined the usability of this method as an epidemiological marker of virulent serovar 1a strains, and compared it to ribotyping and PFGE. The results are shown in Table 22.1. Fifty-three isolates from the same farm belonged to the same RFLP type specific to this district. However, by ribotyping and PFGE, isolates from arthritis tended to be divided further and this made it difficult to find epidemiological relationship among these isolates. Although this RFLP is laborious, we think it will be a useful tool for the epidemiological study of the virulent strains of serovar 1a, the most important in swine erysipelas. We routinely examine *Erysipelothrix* isolates as follows; at first determine the serovar, in case of serovar 1a strains

Table 22.1 Comparison of RFLP, PFGE, and ribotyping on 53 strains of virulent serovar 1a isolated from four farms in the same district

				PFGE type					Ribotype						
Farm	RFLP type	Disease type	Year	E	L	M	N	T	1	2	3	6	7	11	Total
OA	CB3	Septicemia	1998	1								1			1
			1999	2								2			2
		Urticaria	1998	2								2			2
			1999	2								2			2
		Endocarditis	1999	3								3			3
		Arthritis	1999	3	6	3	2		6	2	1	3		2	14
IW	CB3	Urticaria	1999	1								1			1
		Arthritis	1999		1	2			1		1			1	3
	C2B3	Arthritis	1999	1	1				1			1			2
AT	CB3	Endocarditis	2001		2				1				1		2
		Arthritis	2001		1		1			2					2
YK	FF	Urticaria	2004					1					1		1
			2005					7					7		7
		Arthritis	2005					10	4				6		10
			2006					1					1		1
Total				15	11	5	3	19	13	4	2	15	16	3	53

RFLP types were determined by *Eco*RV and *Hind* III and shown side by side. Number in the table means the number of isolates. RFLP types CB3, C2B3, and FF were found only in this district of Japan and C2B3 and FF were only in these two farms. Isolates from septicemia, urticarial type, and endocarditis showed uniform genotype even by PFGE and ribotyping. To the contrary, isolates from arthritis had a tendency to be divided into a variety of genotypes by these methods, and this made it difficult to find their relationship

differentiate virulent strain from vaccine strain by RAPD, and then apply RFLP to virulent strains of serovar 1a to examine epidemiological relationship.

22.1.2.8 DNA Sequencing

Species Identification by DNA Sequencing

For the identification of bacterial isolates, the sequence of 16S rDNA is used frequently. However, bacterial classification is based on DNA homology and it is well known that in some cases the nucleotide sequence of 16S rDNA not necessarily correlated to bacterial species. For example, the 16S rDNA sequences of *E. rhusiopathiae* and strain 715 of *E.* sp.-2 were identical. The 16S rDNA sequence of unestablished species "*E. muris*" are listed in GenBank. Ahrné et al. [22] determined partial sequence of 16S rDNA of nine strains of *Erysipelothrix*, and found a third species and also the inconsistency between species and serovar.

Strain Identification by DNA Sequencing

Nagai et al. [41] reported sequence variation of *spaA* gene among ten isolates of serovar 1a of RAPD type 1-2, specific to Japanese live vaccine strain Koganei 65-0.15, and concluded that only three strains showed the identical sequence to Koganei 65-0.15.

22.1.2.9 Molecular Typing of *Erysipelothrix* in the Future

Many kinds of molecular typing methods have been developed for the identification and discrimination of bacteria especially in human pathogens, some are automated and some have worldwide database. To the contrary, molecular typing of *Erysipelothrix* are not fully evaluated for the practical epidemiological use except for genus and species identification. The most important thing for the epidemiological study is the isolation or detection of the causative bacteria from both infected animals and suspected sources. Molecular typing should present obvious data confirming the connection of these isolates.

In case of swine erysipelas, species identification is not always necessary because almost all of the isolates are *E. rhusiopathiae*. Instead, serotyping gives important information because most of erysipelas isolates belongs to serovars 1 and 2 and only strains of serovar 1a cause highly infectious septicemia [7, 17]. The true mechanism why serovar 1a is the most virulent remains unknown, molecular typing will be an important tool for strain discrimination of *Erysipelothrix* in combination with serotyping, especially among isolates of *E. rhusiopathiae* of serovars 1 and 2.

In *E. rhusiopathiae,* several genes, associated or suspected to be associated to virulence or protection, were identified and sequenced, e.g. capsule synthesis [31],

hyarulonidase [42], hemolysin [40], adhesion [43], tetracycline resistance [44], surface protective antigen A (SpaA) [34], SpaC [45], sialidase (neuraminidase) (Imada, unpublished, GenBank AB019122), and immunodominant cell wall lipoprotein (Anitori, unpublished, GenBank U52850), etc. A multicopy gene sequence neighboring to putative transposase gene was also found [17]. Such genes may be useful molecular typing targets. In general nucleotide sequencing has great merit to be analyzed by computer and easily compared to the data by other researchers. So in near future combination of PCR and nucleotide sequencing will improve molecular typing methods of *Erysipelothrix*.

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Part VI
Pathogens Causing Healthcare-Associated Infection

Chapter 23
Staphylococci

Jodi A. Lindsay

23.1 Introduction

The staphylococci are Gram-positive cocci that normally live on the skin and mucous membranes of mammals and birds. There are over 30 species described, but the major pathogen is *Staphylococcus aureus*. There is rarely reason to type the other species of staphylococci, so this chapter will focus primarily on *S. aureus* and briefly discuss other species at the end.

S. aureus is carried in the nares of the nose in about a quarter of the healthy human population. It can also be found in the throat, axillae, groin, and intestinal tract. *S. aureus* can also survive on skin for short periods and can survive desiccation on inanimate surfaces for months. It does not normally penetrate skin on its own, but if delivered into a breach of the skin such as wound or catheter site, it is capable of causing minor through to fatal infections. Patients who are already immunocompromised, elderly, and diabetic are at higher risk of infection. *S. aureus* can seed to other tissues and therefore cause bacteraemia, pneumonia, abscess, arthritis, osteomyelitis, endocarditis, meningitis, conjunctivitis, etc. In hospitals, antibiotic-resistant strains, notably MRSA, are more prevalent. New strains of MRSA are increasingly found in the community (CA-MRSA) that cause invasive skin infection in young and healthy populations [1].

23.2 Why Type?

S. aureus strains are typed for two main reasons. Firstly, at the local level, to identify clones that have unique pathogenic or epidemiological characteristics. Identification

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of these isolates can help to predict disease prognosis and optimise management, as well as identify epidemiological patterns and strain spread in a local environment. An example is the rapid identification of typical USA300 CA-MRSA clones that cause unique types of infection in some geographical areas and may require different patient management [2]. Local typing can also be useful for the identification of the scale and scope of a local outbreak, its likely source, and behaviours that contribute to spread, so that infection control strategies can be targeted to prevent further disease. An example may be the investigation of an outbreak of an epidemic MRSA in a hospital intensive care unit, where typing can help identify which patients were part of the outbreak, confirmation of likely sources of the outbreak, and supporting evidence that the outbreak has been stopped [3, 4].

The second reason to type is usually performed by a national or international reference laboratory, investigating large-scale evolution and spread of clonal types over large geographical areas and time. These studies are particularly useful if additional data, such as antibiotic resistance, disease, and patient details, are also collected as it can help identify shifts in key pathogenic behaviour. Examples include the emergence and spread of new epidemic MRSA clones, their spread across national borders or from unique sources such as livestock (LA-MRSA), the association of PVL toxin with necrotic pneumonia, and TSST-1 toxin with toxic shock syndrome [5–9]. This greater understanding of how *S. aureus* cause disease, evolve, and spread contributes to preventative strategies, such as screening or optimised antibiotic prescribing, and may also contribute to future diagnostics and therapeutics.

Historically, phenotypic methods were used to type *S. aureus*, most notably phage typing. Molecular methods became common from the mid- to late-1990s, particularly pulsed-field gel electrophoresis (PFGE), although these methods rely on the generation of band patterns that can be difficult to reproduce. More recently, typing methods have been dominated by sequence-based analysis, including MLST and spa typing, which are easy to compare between laboratories internationally. At the time of writing, there are new technologies and discoveries being made that are likely to have a major impact on *S. aureus* typing in the near future, including the introduction of methods that identify multiple clone-specific genes, perhaps as early as during diagnosis.

Each method has advantages and disadvantages, which will be discussed, and are briefly summarised in Table 23.1. The ideal typing method is discriminatory enough to identify isolates that are truly different, but not liable to suggesting isolates are different due to minor genome instabilities. The data generated should be accurate and reproducible and available in a format that is easily compared between remote laboratories. It should be inexpensive so that enough strains can be typed to draw useful conclusions without having to justify cost. Similarly, it should use equipment that is inexpensive or can be rented for a reasonable fee. The test should be simple to perform so as to reduce human errors and to ensure that the widest number of users have access to the method. Rapid tests are useful for investigating outbreaks, where only timely results can influence outbreak management—this depends not only on the speed of test but also on logistics of transporting specimens and reporting

Table 23.1 Simplified comparison of the advantages of each *S. aureus* typing method

	Detect lineage	Detect MGE	Simple to perform	Reproducible	Low cost	Low-cost equipment	Rapid
MLST[a]	+++	–	++	+++	+	++	+
Spa typing[a]	+++	–	++	+++	++	++	+
Microarray (whole genome)	+++	+++	–	+++	–	–	–
Mini-microarray	+++	++	++	+++	++	+	+
RM test	++	–	+++	+++	+++	+++	++
PFGE	+	+	–	+	+	+	–
Phage	+	+	++	–	+++	+++	++
SCCmec PCR	–	+	++	+++	+++	+++	++
Antibiotic resistance	–	++	+++	++	+++	+++	++
Toxin PCR	–	++	++	++	+++	+++	++

+++ an important advantage, ++ competitive, + acceptable, – disadvantage

[a]If MLST or spa typing is to be used for hundreds of strains, the cost per test can be lowered by purchasing or renting your own sequencer

results, and this is more likely if the test is simple enough to perform in-house rather than at specialist reference laboratories. The method you choose will depend on why you wish to type the isolates, as well as logistical factors such as cost, speed, and skill involved.

To interpret the data generated by *S. aureus* typing methods, it is essential to understand how *S. aureus* genomes vary, how *S. aureus* populations are structured, how they are evolving, and how stable genomes are. This has become possible in the last few years because of whole genome sequencing projects, large epidemiological studies with molecular typing methods, and whole genome comparative studies with multi-strain microarrays.

23.3 How Do *S. aureus* Isolates and Their Genomes Vary?

S. aureus populations are continually evolving in response to antibiotics, various hosts, and immune attack. *S. aureus* isolates can be grouped into dominant lineages and some minor lineages. In humans, approximately 10 lineages predominate; they are clonal complexes (CC1, CC5, CC8, CC12, CC15, CC22, CC25, CC30, CC45, and CC51 [10]). Each lineage is very different from the others and is evolving independently. Each lineage has a unique and stable combination of hundreds of genes, notably the genes encoding surface proteins [11].

Within each lineage, minor variations in the core genome can occur. Single-nucleotide polymorphisms (SNPs) can occur, and this is the basis of MLST typing into ST rather than CC/lineage groups (see below). One whole genome sequencing study has estimated that in hospitals SNPs may occur on average once every 6 weeks

[12]. Other studies in patients have identified dozens of SNPs developing in the infecting isolate during the course of chronic infection; often they are selected because they enhance resistance to antibiotic therapy [13, 14].

The second major way that *S. aureus* strains vary is their carriage of MGEs. MGEs include bacteriophage, *S. aureus* pathogenicity islands (SaPI), plasmids, transposons, and staphylococcal cassette chromosomes (SCCs). These elements often encode important toxins, virulence factors, and antibiotic resistance genes [15]. Based on sequencing and microarray studies, most strains carry between 3 and 12 MGEs. MGEs are highly variable, each being composed of a mosaic of gene fragments found in other MGEs, but they can be grouped into families based on their major replicative machinery [16]. Each MGE has unique properties, but many are highly unstable and move into and out of bacteria at extremely high frequency [17, 18]. MGE movement can also be documented during the course of infection within a single patient [19–21]. Examples of the genes found on bacteriophage and SaPI are toxic shock syndrome toxin, enterotoxins A, B, C, K, and Q, chemotaxis inhibitory protein, staphylokinase, staphylococcal complement inhibitor, exfoliative toxins, and Panton–Valentine leukocidin (PVL) [15]. Plasmids and transposons can carry genes encoding resistance to antibiotics such as tetracyclines, aminoglycosides, macrolides, fusidic acid, mupirocin, β-lactams, and, more recently, glycopeptides [22].

The SSC element that encodes the mecA gene for resistance to β-lactamase-resistant β-lactams (such as methicillin) is an exception. Although it is mobile, it is transferred inefficiently and is generally stably integrated into the *orfX* gene. There are several types that are widespread, and the acquisition of each *SCCmec* type has been used as evidence of independent evolution of particular MRSA clones [23].

Since the *S. aureus* genome varies in distinct ways, it is very important to consider whether the typing method you wish to use can actually detect the variation you should be looking for and therefore that you are interpreting the results correctly. In fact, most typing methods are best at proving two isolates are unrelated. Proving two isolates are identical or closely related is impossible without whole genome sequencing and testing the stability of any changes, which is obviously not feasible for routine typing. Therefore, compromises must be made when choosing a method. Knowing your local *S. aureus* epidemiology can help enormously to identify which is the most suitable method.

23.4 Global Epidemiology

In humans, about 10 lineages of MSSA predominate, and all carriage strains seem capable of causing disease [15]. Increasingly, studies from outside of the UK suggest that there are geographical differences in the predominant *S. aureus* lineages [24–28]. Animals can also be carriers of *S. aureus* and can be infected. Companion animals such as dogs and cats are often affected by human lineages [29], while cows and other ruminants, and pigs have their own lineages, and chickens carry a variant of the CC5 lineage [30–32].

The biggest epidemiological shift in the last 50 years has been the acquisition of methicillin resistance due to the mecA gene carried on *SCCmec* elements [1]. *SCCmec* have moved into the lineages CC5, CC8, CC22, CC30, and CC45 and become successful in hospitals [23]. An interesting study of CC5 MRSA in Germany proved that the *SCCmec* element was moving into local CC5 MSSA isolates generating multiple new clones [33], although this may not be true for other lineages. There is a marked geographical difference in the distribution of lineages in different geographical areas, and in most hospitals, only one or two clones dominate [8, 34]. Some countries have reported shifts in the dominant lineages over time [35–37].

CA-MRSA emerged outside of hospitals and predominantly in the lineages CC1 and CC8. However, some less common lineages have acquired both the *SCCmec*IV and PVL toxin on a bacteriophage and spread rapidly, including ST80 and ST59 [38]. More recently, an MRSA clone associated with pig farming has emerged to cause infection in humans (CC398) [39].

The important message here is that there is substantial variation in *S. aureus* and MRSA types depending on geography, clinical setting, and time. It is therefore vital to understand your local epidemiology before choosing a method for typing and interpreting your results. For example, the major MRSA clones in UK hospitals are CC22 (MRSA-15) and CC30 (ST36, MRSA-16). When investigating an outbreak of MRSA in a UK hospital, a typing method that detects only lineage is not going to be very useful on its own, and methods that target MGEs (or SNPs) are also required.

23.5 Typing Methods

S. aureus typing methods are now all molecular and based on DNA sequence variation. They usually rely on the generation of a pure culture of *S. aureus* as the first step. In the clinical setting, this usually means that a specimen is plated onto a primary plate and then a single colony is chosen and plated again onto a fresh agar plate or is grown in broth. These two steps usually take 1 day each. The pure culture then has its DNA extracted—a relatively simple and rapid method suitable for PCR steps (see MLST) or the commercially available automated magnetic bead methods (e.g. MagNA Pure) [40]. Alternatively, if good-quality DNA is required for microarrays or if the DNA is to be stored, then Qiagen columns or the Edge system is recommended [19, 34]. In the clinical lab, if a rapid MRSA detection system using PCR is used (such as BD GeneOhm MRSA or Cephaid Xpert™ MRSA) [41], then the DNA extracted may be suitable—however, this DNA is not necessarily from a single bacterial isolate that can complicate data interpretation. Many typing methods rely on PCR to generate bands for sizing on agarose gels or for subsequent sequencing, and most standard methods are suitable. A step-by-step example is provided in Lindsay and Sung [42].

Ideally, typing methods should identify both lineage and carriage of a range of MGE. Most methods listed below achieve only one of these, and so they may be

used combined with another method. Microarrays and whole genome sequencing have the potential to do both, but microarrays have only recently been developed for routine typing and are still improving. Note that for local typing of an MRSA outbreak, such as in the hospital setting, there may be only one or two dominant clones of MRSA in the hospital, so a lineage method alone will not be sufficient to discriminate between variants.

23.5.1 Detection of Lineages

Lineage detection uses methods that target variation in the relatively stable genes that are conserved within lineages but vary significantly between lineages. MLST is the gold standard, but spa typing is almost as useful and uses only one-seventh of the sequencing reactions and is therefore easier and cheaper to perform. A simple PCR test has also been developed, and microarrays are also useful.

23.5.1.1 Multi-Locus Sequence Typing

MLST involves the PCR amplification and sequencing of seven "housekeeping" genes; these genes are found in all isolates, and the sequences are relatively conserved because these genes are involved in basic metabolism. However, point mutations and minor variations are found, and they correspond closely with lineage. For typing, the sequence of each gene is compared using a Web database (www.mlst.net), and each sequence variant has a unique number assigned [43]. Each strain is then defined by a series of seven numbers, which is then assigned a sequence type (ST) number. There are thousands of strains that have been sequence typed. If two isolates share at least five out of seven numbers, they are assigned to the same clonal complex (CC), and CC is essentially the same as lineage. This can be visualised using free software called eBURST [44].

MLST is an excellent method for assigning lineage. The significance of ST types within a lineage/CC is less clear. There are some cases where an ST type correlates strongly with a unique phenotype, such as the ST36 variant of CC30, which is unique to epidemic MRSA strains found in the UK that have spread to other parts of Europe and around the world [23]. In other cases, ST variants within a lineage do not seem to represent true evolutionary branches [11]. Possibly, this is because a single point mutation is sufficient to generate a different "ST type", which is not truly a new evolutionary branch, or because isolates within a lineage frequently exchange DNA [45].

MLST uses pure DNA, seven PCR reactions, and sequencing of each PCR product in both directions [43]. PCR is simple and cheap. If you are only typing a small number of strains and do not have your own sequencer, the sequencing reactions are outsourced and rapidly become expensive. Alternatively, if large numbers of strains are routinely MLST typed, a sequencing machine can be purchased or hired, and

bulk reagents used so the cost per strain is modest. The data are reproducible, but can be severely affected by sequencing errors leading to incorrect ST assignment, although lineage assignment would remain correct. The data are in a format that is easily reported and comparable with other laboratories. MLST is widely used by research laboratories and reference laboratories as the gold standard when publishing epidemiological data.

23.5.1.2 spa Typing

The *spa* gene encodes protein A, a protein anchored to the cell wall that binds non-specifically to the F_c portion of antibodies, and is found in all isolates of *S. aureus*. The "X" region in the C terminal end of the *spa* gene varies according to lineage, and isolates from different lineages have different amino acid combinations in multiple repeat patterns [46–48]. Specifically, most strains have an X region of between 2 and 18 different short sequence repeat regions (SSRRs), and each SSRR is around 24 bp each. For typing purposes, each unique SSRR sequence has been assigned an "r" number, e.g. r01, r02. Each *S. aureus* isolate is assigned a series of 2–18 "r" numbers in order, and then this defines the *spa* type number [48]. *spa* type numbers are reported in the format "t001," "t002", etc., and several thousands have been described so far. The *spa* typing method requires pure DNA, PCR amplification of a region of the *spa* gene, sequencing of the region in both directions, and comparison of the sequence to a public database, which contains all the SSRR type numbers and *spa* type numbers (www.spaserver.ridom.de) [48]. Software called BURP can be used to show the relatedness of each *spa* type [49].

spa typing is a reliable way to assign lineage. When assigning a *spa* type to a lineage, the matching MLST CC- or ST-type numbers are used, and for practical purposes tables of matching *spa* and MLST types are available (www.spaserver.ridom.de). Isolates of the same lineage have related *spa* types; for example, *spa* types of t001, t002, t003, and t010 all belong to lineage CC5 and have similar sequences of SSRR "r" numbers, varying only in minor deletions of an SSRR, duplications of an SSRR, or point mutations causing a change in an SSRR "r" number. *spa* type variation between isolates within the same lineage can be used for typing purposes, especially when investigating outbreaks. However, it cannot be assumed that two isolates within the same lineage with slightly different SSRR "r" numbers are epidemiologically unrelated. The stability of the *spa* region during outbreaks is relatively unknown, and *spa* variants do not always match variants identified using MLST [50, 51] or whole genome sequencing [52]. However, there are examples where lineage variants correlate well with epidemiological spread, suggesting there are occasions when variations of the gene are stable. Therefore, care should be taken when interpreting lineage "variants" to ensure that they are only considered significant when backed up with robust epidemiological data.

spa typing is reproducible [8], although sequencing errors are possible. The results are easily compared between laboratories using standard *spa* type numbers from the database. The cost of PCR is inexpensive, the equipment required is widely

available and inexpensive, and the reaction takes only a few hours. The equipment for sequencing is not standard, and as for MLST, if a lot of strains are to be typed, a sequencer should be purchased. Alternatively, for those typing a small number of isolates, commercial companies will sequence purified PCR products for a modest fee, and the results will be available within a week. Technically, this is not a difficult test, and only some experience interpreting the *spa* types is necessary.

spa typing is rapidly becoming the typing method of choice for reference and research laboratories as it is cheaper and simpler than MLST, equally discriminatory, reproducible, and easily reportable [51, 53]. Although *spa* typing relies on only one gene, which may be unstable, MLST typing also relies on variation in one of seven genes to assign a unique ST. *Spa* typing is most valuable when combined with other methods, especially those that can detect MGE variation [50, 51].

23.5.1.3 Microarray

Microarrays are solid supports (usually glass slides) with different DNA spots printed or synthesised onto the support in known order. Microarrays can carry only a few hundred spots or up to hundreds of thousands. They are used to interrogate complex mixtures of nucleic acid, including DNA from a pure culture for typing purposes. The bacterial DNA is labelled and hybridised to the slide so that unwound DNA strands with complementary base pairs will bind specifically to their matching spots, such that only those DNA spots representing genes found in the bacterial DNA will become labelled. The slide is then scanned to see which spots are labelled and the data analysed and compared to control strains and known populations. There are an infinite variety of microarrays depending on the number and types of DNA spots on the array, the solid support, labelling system, controls for the spots, hybridisation conditions, detection system, and data analysis methods [54]. For this reason, only microarrays that have been thoroughly validated for typing purposes using well-characterised (sequenced) strains of *S. aureus* should be used. For this discussion, there are two main types of microarrays to consider—comprehensive multi-strain *S. aureus* microarrays and those specifically designed for *S. aureus* typing.

Comprehensive multi-strain microarrays are excellent for assigning strains to lineage based on the presence or absence of hundreds of lineage-defining genes, such as surface proteins, regulators, hsdS variants, and exotoxins [11, 55]. In addition, they are an excellent method for identifying the presence and absence of a wide variety of MGE (see below). However, such microarrays are relatively expensive—arrays have to be purchased or printed, and the expense of labelling methods, especially the Cy dyes, restrict this technology to the research environment. Scanners are modestly expensive and so is software for analysing data. Microarray data are reproducible and take 1–2 days, but are very technically demanding, particularly to interpret the data. A large volume of data is generated so it is difficult to report, although it can be simplified to lineage and presence or absence of major toxins and resistance genes. For publication purposes, data should be MIAME compliant and deposited in a public database, which is a substantial amount of work [4, 56–60].

Such microarrays are invaluable for asking important epidemiological and biological questions about evolution, pathogenicity, host interactions, and the role of key genes in infection.

Several microarrays specifically designed for *S. aureus* typing have been developed [61–70]. At present, only one of them is designed specifically to identify lineage directly [71], but this should require only minor modifications to other microarrays, such as incorporating hsdS probes [34]. These microarrays currently focus on the presence or absence of putative virulence genes although the typing significance of these genes is often not clear. Companies also make it possible to design your own microarray to your own specifications, and this should be considered by reference laboratories who specialise in discriminating between *S. aureus* types in their local area. This is feasible now that so many *S. aureus* isolates have been fully sequenced [72]. Specific equipment for scanning microarrays and software for interpreting data are necessary, but these can be used for bacteria other than *S. aureus*. The cost of individual microarrays or "strips" of microarrays is low compared to other typing methods. The technique requires some expertise particularly in interpreting the data. *S. aureus* typing microarrays have the potential to be highly reproducible and reportable. There is likely to be a rapid improvement in technology and usefulness of these types of tests in the near future.

23.5.1.4 Restriction–Modification Test

The restriction–modification (RM) test is a simple PCR test to identify variants of the hsdS gene, which define the major MRSA lineages. All *S. aureus* isolates carry a type I restriction modification system called Sau1, and this consists of five genes, hsdR (restriction), two hsdM (modification), and two hsdS (specificity) [45]. RM systems identify foreign DNA at specific sequences, digest the DNA, and protect the host bacterium from bacteriophage. To protect the bacterium's own DNA, the same specific sequences are recognised and modified. *S. aureus* isolates belonging to the same lineage have the same hsdS sequences, but isolates from different lineages have different hsdS sequences. This means that DNA from different lineages is recognised as foreign and therefore exchanged between strains at lower frequency than within the same lineage. This controls the independent evolution of lineages and the spread of MGE [45, 73]. The test involves isolating DNA, up to three multiplex PCR reactions, which are separated on a standard agarose gel, and assigning a lineage based on a very simple test for band presence or absence.

The RM test is currently designed only to identify the major MRSA lineages, CC1, CC5, CC8/ST239, CC22, CC30, and CC45 [34, 73]. CC8 and ST239 can be distinguished by capsule-type PCR ([19], Cockfield and Lindsay unpublished) or by a PCR test based on the junction of the large recombination of a CC8 and CC30 strain that leads to the emergence of ST239 [74]. A new RM test that identifies ST398, the prevalent livestock-associated MRSA, has recently been validated [75]. The RM test will be expanded to include more lineages in the future.

RM typing is designed to be simple, rapid, and inexpensive. The results are reproducible and easily reported. It is particularly useful for typing very large numbers of isolates for epidemiological studies and can be easily automated or scaled up. It is a relatively new test, but its use is becoming more popular.

23.5.1.5 Pulsed-Field Gel Electrophoresis

PFGE was developed in the mid-1990s and was the first useful molecular test for typing *S. aureus*. It became popular with typing laboratories all over the world and is still widely used. PFGE patterns in experienced hands can provide useful information about dominant clones. PFGE cannot be used to define a lineage, but can provide clues if your local epidemiology is known, as well as some information about MGE distribution.

PFGE involves extraction of pure DNA using a specific process that catches the DNA in a plug of agarose, digestion of the DNA with SmaI, a site-specific restriction enzyme that cuts the *S. aureus* genome infrequently, and separation of the DNA fragments on an agarose gel in a specialised gel apparatus that applies electrical current in two or three directions in changing pulses and allows separation of much larger fragments than standard agarose gels. The gel is then stained, and the pattern of bands is photographed and compared using digital software.

PFGE identifies "patterns" of DNA fragments based on their size but does not identify what any of the fragments are. The insertion or deletion of an MGE is sufficient to cause a change to a PFGE band profile. For example, if a strain picks up a bacteriophage that integrates into the chromosome, and the phage contains two copies of the SmaI recognition site, the pattern will have two bands that differ in size and one extra band. Alternatively, a point mutation in a SmaI site can lead to changes in band size or number. In practice, it is acknowledged that during an outbreak situation, strains that vary in up to four bands are probably related [76], so interpretation of data is complicated. PFGE does not actually identify lineage and benefits from being combined with a method that determines lineage accurately [51]. However, in highly experience hands and when the local epidemiology is known, standard pattern types can be recognised as belonging to major clones, which in turn belong to known lineages. For example, the CDC in Atlanta uses PFGE to identify about 10 dominant clonal types of MRSA in the USA [77]. In the local typing situation (see below), where only one or two MRSA clones dominate, small variations in patterns can be useful. However, care should be taken to interpret the data, since relatively minor acquisition or loss of MGE can lead to significant band variation, yet this can occur in a single patient during the course of infection [20]. Furthermore, unrelated strains can have quite similar PFGE profiles [59].

PFGE requires investment in specific equipment, including a CHEF electrophoresis system, data documentation system, and software for comparing profiles. The reagents for each test are then modestly priced. However, the major drawback to PFGE is that the band patterns are difficult to reproduce unless the user is highly experienced. Therefore, standard protocols that carefully define every component

reagent and step have been developed in order to ensure that the data are reproducible and comparable between laboratories [78]. In cases where two strains from different gels are being compared, it may be necessary to run a further gel to compare them to each other. The patterns are not easily described without pictures and therefore it is difficult to compare results from different laboratories. The method itself is relatively labour intensive compared to other typing methods.

23.5.1.6 Other Methods

Prior to genetic tests such as PFGE, the major *S. aureus* typing method was bacteriophage typing [79]. Phage typing has not been formally compared to the newest typing methods, but it clearly provides clues to lineage and phage distribution without defining either. A set of bacteriophage, each grown on a specific *S. aureus* host strain, was spotted onto the strain to be tested and the pattern of phage lysis versus non-lysis to each phage used to discriminate between strains. Strains were typically classified into three or four major groups with the "international" typing set, and these groups probably correlate well with lineage, although this method cannot be used to define the lineage. In addition, significant variation within each group is seen, and this is probably correlated with the presence or absence of other bacteriophage in the host strain's genome that prevent lysis with a related phage. There is still a lot that is not understood about how phage patterns are generated.

Phage typing is very inexpensive, rapid (overnight), and simple to perform and interpret. Only a few laboratories in the world still use phage typing and keep sets of phage. It is interesting that many modern MRSA are poorly lysed by the old phage sets [80, 81], making them less useful today.

Several other typing methods have been developed that have potential to rapidly and inexpensively identify lineage. Multiple locus variable tandem repeat analysis or variable number tandem repeats involves PCR of several surface protein genes that are then digested with restriction enzymes and the band sizes compared as patterns. It can detect lineages without defining them, but the correlation is not exact and comparing patterns between laboratories is difficult [82–84]. A similar method, staphylococcal interspersed repeat units, involves the sequencing of seven repeat regions scattered throughout the *S. aureus* chromosome. It is a useful method of separating strains of different lineage, but is not widely used and there are no support tools to assign lineages [85]. Amplified fragment length polymorphism involves the use of random primers to PCR random fragments of DNA and the patterns generated are compared. Although useful for rapid screening of large populations, it can identify only some of the lineages reliably [86]. A rapid version of MLST using mass spectrometry to detect variants has also been developed [87].

Other PCR tests are sometimes reported. The accessory gene regulator (agr) is the major regulator of toxin production in vitro, and there are four different types that can be discriminated by PCR: I, II, III, and IV [88]. However, their distribution correlates exactly with lineage [11], so the method is less sensitive than other

lineage-typing methods. Similarly, capsule type also correlates exactly with lineage [11, 89], and there are only two types: 5 and 8.

23.5.2 Detection of MGE

23.5.2.1 *SCCmec*

SCCmec typing is very popular as the *SCCmec* element is generally stable, and combined with a lineage detection method, it is useful for the identification of well-known epidemic MRSA types [23]. There are eight major types of *SCCmec*, types I–VIII, although new types and variants are increasingly being described. Within each type, there is variation, suggesting recombination and movement of elements such as transposons and plasmids embedded into the *SCCmec* element. Note that SCC elements without *mecA* genes exist, but are rare in methicillin-susceptible *S. aureus* [90]. Typing is dependent on PCR reactions that detect variation in the ccr recombinase genes and the *mecA* region, and use a potentially large number of primer pairs, but the methods are relatively straight forward, simple to perform, and inexpensive ([91, 92], www.staphylococcus.net/). This method is useful if you wish to assign an MRSA isolate to a major MRSA clonal type. However, it can be less useful in investigating local outbreaks, as the *SCCmec* elements are generally stable.

23.5.2.2 Microarray

Microarrays were described above for their ability to detect lineage. However, they are also particularly useful in detecting MGE variation because multiple MGE types and variants can be detected in a single experiment [93]. It is important to keep in mind that genes can be detected only if they are spotted on the microarray. Simple microarrays with a limited number of MGE spots are easier to interpret but less comprehensive than complex whole genome microarrays.

In general, there is no standard way to interpret microarray data. The presence or absence of a range of toxin and virulence genes, as well as antibiotic resistance genes found on MGE, is a logical place to start. Tables of various MGE types and their distribution into families based on integration genes and replication loci can also be very useful [16]. In practice, if two isolates have the same lineage and nearly identical combinations of MGE genes, as well as a strong epidemiological link, then they are likely to be the "same" strain. Two isolates that vary in genes likely found on only one MGE (e.g. one plasmid or one bacteriophage) and are also likely to be related but can be distinguished. Those with more variable MGE content are less likely to be epidemiologically related [4, 57–60].

23.5.2.3 PCR for Toxins and Other MGE Genes

There is currently no standard method for reporting the presence or absence of toxin genes encoded on MGE by PCR for typing purposes. However, increasingly, papers reference these reactions to justify their assignment of a strain to a particular clone or to investigate associations with particular diseases [94]. The most common example is the PVL toxin. PVL is relatively rare in *S. aureus*, but CA-MRSA nearly always carries it, and it is a useful marker for these strains [95]. Its role in CA-MRSA disease is controversial [96]. Other toxins with a potential role in pathogenicity can be useful to identify, especially if their detection has benefits for the patient or for understanding an outbreak, such as the food poisoning toxins [97], exfoliative toxins [98], and toxic shock syndrome toxin [95]. Such toxins may be unstable as they are found on MGE, and this should be considered when interpreting data, especially if only a few PCRs are used.

23.5.2.4 Antibiotic Resistance

Phenotypic antibiotic susceptibility testing is routinely performed in diagnostic laboratories on all *S. aureus* considered to be clinically significant. The results are generally available within 2 days of *S. aureus* diagnosis and are used to support the choice of antibiotic prescribed. There are highly standardised methods for identifying resistance using phenotypic methods, such as disc testing, automated broth testing, and E-tests, and published by organisations such as BSAC (http://www.bsac.org.uk/) and CLSI (formerly NCCLS). There is no standard method used by all diagnostic laboratories. Whichever method is chosen, it is very simple, inexpensive, and rapid.

Antibiotic testing played a part in the older typing methods prior to genetic tests. However, interpretation was difficult and unhelpful. Recently, we have been revisiting these tests in combination with lineage tests and find the combination much more useful. There is generally a wide range of resistance patterns to the major antibiotics, even within epidemic MRSA clones. It is therefore a potentially useful method for discriminating isolates during an outbreak (Budd and Lindsay, unpublished).

23.6 Other Staphylococcal Species

About 12 other species of staphylococci are found colonising the skin and mucous membranes of humans. They are all less virulent than *S. aureus*, but all are capable of causing disease, particularly in immunocompromised patients with prosthetic implants. They are often termed coagulase-negative staphylococci, and the most common skin coloniser and pathogen is *S. epidermidis*. Antibiotic resistance in all human staphylococcal species is common, especially methicillin resistance.

However, there is rarely a clinical reason to type *S. epidermidis* or other staphylococci in the hospital setting, as outbreaks are rare and disease is generally due to host immune factors. Large-scale epidemiological studies have been described recently, and an MLST typing method described for *S. epidermidis* [99]. *SCCmec* typing is also applied as many of these elements likely arose in coagulase-negative species [100]. Interestingly, disease isolates of *S. epidermidis* are predominantly from one particular lineage and have the ability to produce slime [101].

In dogs, the major species that causes disease is *S. pseudintermedius*. Multidrug-resistant isolates are increasingly being described, and MLST, *spa* typing, PFGE, and *SCCmec* typing have also been developed [102].

23.7 Future

In the future, we are likely to see technologies developed that allow rapid and inexpensive typing of *S. aureus* in real time in the clinic. Already there are technologies that can identify pathogen species directly from clinical specimens using microarray detection [103–106]. While mixed populations of bacteria in specimens can make direct typing troublesome to interpret, these technologies are ideal for investigating isolated colonies on selective agar, even after minimal growth.

We are also likely to see improvement of mini-microarrays, particularly in designs that are adapted for different geographical regions. When investigating an MRSA outbreak in a hospital where only one or two MRSA predominate, the array needs to be focused on the MGE present in those clones. Hopefully, more commercial suppliers will enter the marketplace in the near future, automating methods and bringing down costs.

With rapid progress made in the DNA sequencing arena, there is also the possibility that *S. aureus* strain typing in the future will routinely involve whole genome sequencing [12]. This will be the ultimate typing tool and will require substantial progress in cost reduction and developing software able to rapidly compare and contrast the whole sequences of individual bacteria.

When typing can be performed in real time and the results fed back to the clinician, we are likely to see improved management of infections, better recognition of outbreaks, and faster responses to them. We will also recognise more correlations between types of strains and their associations with symptoms, prognosis, susceptible hosts, ability to spread, and geography. This is when we are likely to make the most progress in understanding *S. aureus* genome stability and evolutionary pressures.

23.8 Conclusions

At present, we are in a time of flux, with new typing technologies for *S. aureus* being developed but not fully taken up, and improved technologies on the hori-

zon. In the meantime, the choice of typing method is dependent on the question that is being asked. In general, a comprehensive typing of a strain requires the correct identification of lineage and some understanding of MGE variation. When typing to compare strains in an outbreak setting, such as MRSA in a hospital where only one or two clones dominate, a method that accentuates minor differences is preferred, and microarrays are becoming the method of choice. In the near future, these microarrays will be refined, and the technology will become less expensive and more rapid as the commercial market develops. In the future, the development of bench-top sequencing options, whole genome sequencing combined with customised data analysis software, will become the typing method of choice.

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Chapter 24
Molecular Typing Methods for the Genus *Pseudomonas*

Balázs Libisch

24.1 The Clinical Significance of the Genus *Pseudomonas*

The genus *Pseudomonas* was described by Migula in 1894 as a genus of Gram-negative, polarly flagellated, aerobic rod-shaped microorganisms. The genus has since been subject to repeated taxonomic revisions. Several distantly related species from the old genus of *Pseudomonas* have since been placed in existing or newly defined genera including *Sphingomonas, Burkholderia, Ralstonia, Aminobacter, Acidovorax, Telluria,* and *Stenotrophomonas.* In the past two decades, taxonomic studies using methods for analyzing microorganisms at the molecular level have played a crucial role in improving the classification of the pseudomonads. The RNA group I species, identified based on DNA–RNA hybridization analyses, includes the type species *P. aeruginosa* and other species such as *P. fluorescens*, *P. putida*, and *P. syringae* that are members of a phylogenetically homogeneous group referred to as *Pseudomonas* (sensu stricto). This classification is in agreement with phylogenetic information obtained from 16S rRNA sequence data [1, 2].

The genus *Pseudomonas* is widely distributed in natural and aquatic environments and includes species that can be pathogenic for plants, while others are opportunistic pathogens of animals or humans. Some species of the genus exhibit plant growth-promoting and plant pathogen-suppressing functions and may be exploited for use in biological control. An important property of *Pseudomonas* species or strains is their wide metabolic versatility with regard to the carbon sources that can be utilized. This makes them potential candidates for use in various types of bioremediation. As an example, *Pseudomonas* spp. environmental strains that are able to biodegrade propylene-glycol-based aircraft deicing fluids as a sole carbon source at 4°C could be cultured and isolated from soil samples of the Oslo International

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Airport, which is located on a relatively homogeneous flat sandy area with weakly podsolized soils [3, 4].

P. aeruginosa is the most important human pathogen of this genus causing opportunistic infections in a range of patients. Despite this, other species including *P. fluorescens, P. mendocina, P. putida,* and *P. stutzeri* within the genus *Pseudomonas* can be human pathogens [3].

P. aeruginosa is commonly responsible for nosocomial infections, including surgical-site infections, urinary tract infections, pneumonia, and bloodstream infections. It is recognized as the most important bacterial complication in patients with cystic fibrosis (CF), leading to chronic colonization and intermittent exacerbations ranging from bronchiolitis to acute lung syndrome. *P. aeruginosa* also has properties that make it a particularly problematic bacterium in hospitals, including natural resistance to many drug classes and disinfectants, the ability to acquire resistance through horizontal gene transfer or mutation, and a high virulence potential [5, 6].

Global occurrence rates for non-fermentative, Gram-negative bacilli isolates were published from the SENTRY Antimicrobial Surveillance ProGram medical centers for the period 1997–2001 involving 18,569 strains that included isolates from both nosocomial and community-acquired infections. In this survey, *P. aeruginosa* was the most prevalent non-fermentative, Gram-negative species (64.5%, 11,698 strains) while other *Pseudomonas* spp. isolates constituted 2.8% of all tested isolates, including *P. fluorescens* or *fluorescens/putida* (0.98%), *P. stutzeri* (0.14%), *P. mendocina* (0.02%), and *P. vesicularae* (0.02%) [7].

In the European Prevalence of Infection in Intensive Care Study, up to 28% of nosocomial infections were attributed to *P. aeruginosa*. The incidence of *P. aeruginosa* in bloodstream infections in Europe increased slightly from 5.5 to 6.8% between 1997 and 2002, according to the SENTRY Antimicrobial Surveillance ProGram (1997–2002) where 37 medical centers from 15 European countries participated [5, 8].

24.2 Molecular Identification of *Pseudomonas* spp. Isolates

Phenotypic identification of bacteria isolated from CF patients carries a considerable risk of misidentification. CF-derived isolates often display phenotypic diversity due to loss of pigment production, exopolysaccharide production, and/or synthesis of rough lipopolysaccharide (LPS). Commercial and other phenotype-based identification methods may therefore misidentify *Pseudomonas* spp. isolates due to low metabolic activity and morphological and biochemical variability. Correct identification is inevitable for reliable detection of important pathogens like *P. aeruginosa* particularly with respect to antibiotic therapy, patient prognosis, and infection control for further epidemiological investigations [9].

16S rDNA sequence analysis has long been used as a taxonomic gold standard in determining the phylogenies of bacterial species. Recent advances in molecular ecological taxonomy for the genus *Pseudomonas* in the last few years provided the

necessary DNA sequence data for the design of highly specific PCR primers and protocols for the detection of 16S rRNA genes for molecular identification [2].

Molecular identification methods based on DNA sequencing of 16S rRNA or other genes are not suitable for routine identification of *Pseudomonas* spp. strains in the diagnostic microbiology laboratory due to their high costs, time, and labor requirement. In addition, public DNA databases require an experienced user for interpretation of DNA sequences. However, conventional PCR assays have been developed and used successfully for identification of *P. aeruginosa* in CF patients, and thus this technique may offer an alternative to the laborious methods based on DNA sequencing [9].

LiPuma and colleagues [10] designed 16S rDNA-based PCR assays that provide molecular identification of *P. aeruginosa* and its differentiation from other closely related *Pseudomonas* species. Based on alignment of 16S rDNA sequences available in GenBank, two primer pairs were designed. Primer pair PA-GS-F and PA-GS-R was intended to amplify all *Pseudomonas* species, while the pair PA-SS-F and PA-SS-R was designed to amplify only *P. aeruginosa* sequences (Table 24.1). The latter primers targeted species-specific sequences in 16S rDNA variable regions (V2 and V8), respectively. The utility of this PCR method was demonstrated to accurately identify *P. aeruginosa* among isolates not correctly identified by phenotypic tests.

For isolates testing positive with the genus-level PCR, but negative with the *P. aeruginosa*-specific PCR, identification to the species level can be performed through PCR amplification and subsequent sequencing of the 16S rDNA region using primers UFPL and URPL listed in Table 24.1. By applying this approach, the following non-*P. aeruginosa* species were identified from CF sputum cultures: *P. fluorescens*, *P. lundensis/fragi, P. pseudoalcaligenes, P. stutzeri,* and *P. synxantha*, based on the 16S rDNA sequence analysis [10].

24.3 Molecular Typing Methods for *Pseudomonas* spp. Clinical Isolates

24.3.1 Introduction

P. aeruginosa is characterized by its great biochemical and ecological versatility, and the genome size can vary as much as 30% in different strains. Phenotypic methods that have been reported for typing *P. aeruginosa* include phage, pyocin, and antimicrobial susceptibility typing and serotyping [11, 12]. Phenotypic characterization continues to play an important role in the management of *P. aeruginosa* infections. For example, routine antimicrobial susceptibility testing by clinical microbiology laboratories may identify unique patterns of antimicrobial resistance, which can provide an early warning of potential epidemiological problems among patients [13].

Table 24.1 Oligonucleotide primers for the molecular identification of *Pseudomonas* spp. isolates

Designation	Sequence (5′–3′)	Target gene	Annealing temperature (°C)	Size of product (bp)	Use	Reference
PA-GS-F	GACGGGTGAGTAATGCCTA	16S rDNA, *Pseudomonas* spp.	54	618	PCR amplification and gel electrophoresis	[10]
PA-GS-R	CACTGGTGTTCCTTCCTATA					
PA-SS-F	GGGGGATCTTCGGACCTCA	16S rDNA, *P. aeruginosa*	58	956	PCR amplification and gel electrophoresis	[10]
PA-SS-R	TCCTTAGAGTGCCCACCCG					
UFPL	AGTTTGATCCTGGCTCAG	16S rDNA, bacteria	55	1,490	PCR amplification and sequencing	[10]
URPL	GGTTACCTTGTTACGACTT					

The discriminatory power of a molecular typing technique must be high enough to differentiate unrelated strains from each other, but not so high as to assign isolates of a common lineage (such as an epidemic clone) to distinct genotypes. The discriminatory ability of the different methods is compared in this chapter based on their discriminatory indices (DIs) as described [14]. According to this original reference publication, an index of greater than 0.90 would be necessary if the typing results are to be interpreted with confidence. However, more stringent criteria were proposed in more recent works, where a typing method has enough specificity if the chance that two unrelated strains are grouped in the same type is <5% (<0.05) [15, 16].

Epidemiologically unrelated isolates may have similar or identical genotypes if there is limited genetic diversity within a species or clonal lineage with a wide geographical distribution or if the genotyping method is not sensitive enough to distinguish among the nonclonal isolates. Besides a sufficient discriminatory ability, further attributes of an ideal typing system are high typeability and reproducibility (see Table 24.2), ease of use, low costs, and unambiguous interpretation of the derived data [13]. Table 24.2 summarizes various features of the molecular typing methods discussed in this chapter that focuses only on those ones that seemed to have the widest use in past or current clinical epidemiological studies.

24.3.2 Restriction-Based Methods

24.3.2.1 Restriction Fragment Length Polymorphism (RFLP)

RFLP was the first method to be widely used for the molecular typing of *P. aeruginosa* from patients with CF. In this method, genomic DNA is extracted from the bacterial cells and digested separately with several (such as two or three) restriction enzymes. The obtained DNA digests are then separated by electrophoresis, transferred to a membrane, and hybridized with a radiolabeled probe. The fingerprints created by the size and number of labeled DNA genomic fragments are used to compare the different strains. In the case of *P. aeruginosa*, the most informative data have been derived from studies in which a probe from the gene for exotoxin A (*exoA* or *toxA*) was used [11].

The discriminatory power (DI) of RFLP-*exoA* typing using *Bgl*II, *Sal*I, and *Xho*I restriction enzymes was 0.97 for the combined patterns that is sufficient for discerning between unrelated strains. Some isolates that differed in colony morphology, LPS serotype, and biotype displayed identical RFLP patterns, suggesting that phenotypic variation in *P. aeruginosa* is not necessarily a consequence of genetic heterogeneity. On the other hand, isolates from unrelated patients that were indistinguishable by their serotype, biotype, and antibioGram were assigned by RFLP-*exoA* to different types. A drawback of this method is that approximately 5% of *P. aeruginosa* strains lack *exoA* and thus are not typeable by this approach.

Table 24.2 Comparison of selected molecular typing methods for *P. aeruginosa* by various features

	Typeability[a] (%)	Reproducibility[b] (%)	Discriminatory indices (DI)[c]	Strengths	Weaknesses	References
RFLP (*exoA* probe)	95–100	100	0.97	Ability to discriminate between isolates identical by phenotypic methods	5% of strains lack the *exoA* gene, southern hybridization is labor intensive	[11, 15, 19]
Automated ribotyping (*Pvu*II enzyme)	100	100	0.88–0.93	Rapid typing results, high throughput, profiles are stored in a database	High costs for equipment and reagents, low DI values	[22, 23]
PFGE (*Spe*I enzyme)	95–100	100	0.98–0.997	High DI values, well-defined criteria for interpretation, high reproducibility	Labor intensive and time consuming, costly, results are not readily portable	[24, 30, 31]
AFLP	100	87	0.97	Rapid typing results, highly discriminative	Lower reproducibility than for PFGE, more hands-on time and costs than for RAPD	[43, 45]
RAPD	100	98.5	>0.90[d]	Rapid typing results, low costs, and hands-on time	Low inter-laboratory reproducibility, results are not portable	[47, 48, 52]
MLVA	97.5	100	0.98	Relatively low costs, technical simplicity, portable results between laboratories	7–15 PCR reactions/isolate, exact sizing of PCR fragments may be ambiguous	[31, 53, 55]
MLST	100	100	0.95–0.97	Sound biological basis, unambiguous interpretation, high reproducibility, portable results between laboratories	High costs and hands-on time	[26, 48, 58]

Please see text for further details and references

[a]The percentage of typeable isolates over the total number of typed isolates

[b]The percentage reproducibility of the marker pattern for the same isolate

[c]The DI value is defined in Sect. 3.1

[d]Data correspond to RAPD typing as described [68]. For the method of Campbell et al., no published DI data were available

RFLP typing with the *exoA* probe proved to be useful in investigating the molecular epidemiology of *P. aeruginosa*, but it has now been largely supplanted by pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA analysis (RAPD) (see Sects. 3.2.3. and 3.3.2). Although *exoA* (*toxA*) typing is also indicative of clonal relatedness of strains, this technique was proposed to discriminate less likely between infections caused by variants of the same clonal lineage that persist in different geographical locations [11, 15, 17].

24.3.2.2 Ribotyping

Ribotyping is a type of RFLP when the differences in the location and the number of rRNA genes present in the genome of an organism are analyzed. In principle, DNA restriction fragments are separated by gel electrophoresis, transferred to a membrane, and incubated with a probe specific for a conserved region of the rRNA genes. Ribotyping of *P. aeruginosa* using *Pvu*II restriction enzyme and an rDNA gene probe for hybridization resulted in a lower discriminatory power (DI = 0.956) compared to the RFLP method above using the *exoA* probe (DI = 0.97). Pitt and colleagues were the first to use ribotyping for the study of multiresistant serotype 012 *P. aeruginosa* strains from Europe. Their results agreed with those of outer membrane protein electrophoresis, LPS analysis, and esterase typing in demonstrating uniform features suggestive of a common origin of these strains [15, 18, 19].

In the past decade, automated ribotyping systems, such as the RiboPrinter™ Microbial Characterization System (E.I. duPont de Nemours and Company), became more widely used compared to manual protocols [17]. Such an approach standardizes the technical and interpretive aspects of the procedure and also uses a computer database to compare profiles from a large number of isolates. The system proved easy to use with *P. aeruginosa* isolates, and the total hands-on processing time was estimated at approximately 12 min per isolate. The ability to automatically type 32 isolates in an 8-h period and to compare patterns within a gel and with a large database was a very advantageous feature of this system [20].

For *P. aeruginosa*, the discriminatory power of ribotyping with *Pvu*II restriction enzyme has been claimed to be better compared to that with *Eco*RI [21, 22]. In addition, *Pvu*II has been suggested to be more stable and reproducible than *Eco*RI. For these reasons, *Pvu*II was selected for the analysis of *P. aeruginosa* isolates by automated ribotyping in a molecular surveillance study of European quinolone-resistant clinical isolates of *P. aeruginosa* using the RiboPrinter system [23]. The authors concluded that automated ribotyping had excellent reproducibility, typeability rate, and high-volume capacity. This approach appeared to be a convenient way to rapidly identify and compare the prevalent bacterial clones in distant geographical regions and time points.

With the RiboPrinter system and *Pvu*II enzyme, DI values of 0.88 and 0.93 were reported for *P. aeruginosa* [22, 23]. To investigate the genetic differences between strains belonging to a unique ribogroup further, other typing methods such as PFGE or AFLP were recommended, which are more discriminatory typing systems, but

they are also more time-consuming and of lower throughput. These follow-up methods may be used on selected isolates that are clustered within the same ribogroup [23].

In a comparative study of automated ribotyping, PFGE-, and PCR-based fingerprinting of 64 *P. aeruginosa* isolates, the main drawback of automated ribotyping was found as the high cost of the equipment (approximately 150,000 USD), reagents and maintenance. This was the most expensive technique out of the three molecular typing techniques analyzed in that study [24].

24.3.2.3 Pulsed-Field Gel Electrophoresis (PFGE)

For the molecular typing of *P. aeruginosa*, PFGE or macrorestriction analysis is currently considered the gold standard (or reference) method because of its high level of typeability, reproducibility, and high discriminatory power [11–13, 25, 26].

PFGE is a type of RFLP when the bacterial genome is digested with a rare-cutting restriction endonuclease that generates a smaller number of DNA fragments that can be separated using specialized electrophoresis equipment. This equipment allows the large fragments (usually about 50–500 kbp) to migrate through the gel. The chromosomal DNA is protected from mechanical breakage by immobilization of bacterial cells through embedding them in the agarose matrix as opposed to the manipulation of free DNA. The embedded cells are lysed by use of detergents and enzymes (such as proteinase K), and the released DNA is immobilized in the agarose plugs. After digesting the DNA, the obtained DNA fragments are separated, stained, and the patterns are examined by visual inspection or by computer-assisted methods.

The selection of the restriction enzyme used is species specific and has a key role in determining banding patterns. Currently, macrorestriction using the *Spe*I enzyme can be considered as the gold standard method for *P. aeruginosa* [12]. The PFGE-*Spe*I approach makes use of the rare *Spe*I restriction sites in the *P. aeruginosa* genomes, allowing the comparison of digested genomic DNA profiles with DNA bands ranging in size from about 10 to 700 kbp [15, 27, 28].

This enzyme usually generates 14–25 bands per strain [27, 29], although band numbers up to 37 were also reported [15]. Reproducibility using the *Spe*I enzyme was 100% [30, 31], and typeability ranged between 95 and 100% [15, 24, 30, 32]. For non-typeable strains, autodegradation of DNA was reported most frequently, which could be eliminated in one study by use of HEPES buffer instead of Tris or the addition of 50 mM thiourea in the gel buffer to scavenge reactive Tris radicals [32]. In different studies, PFGE-*Spe*I was determined to have DIs between 0.98 and 0.998 [24, 30, 31].

Digests using other restriction enzymes such as XbaI and DraI provided patterns of 22–35 fragments in the 10- to 350-kb range [33]. The reproducibility using XbaI was about 95% and the discrimination index was 0.966 when including the subtypes in the computation [34]. The XbaI enzyme may generate less clear and well-sepa-

rated bands as compared to *Spe*I, making interpretation of banding patterns less straightforward or unambiguous.

Tenover and colleagues developed general guidelines for the epidemiological interpretation of macrorestriction profiles that became widely accepted and form the basis of subsequent recommendations also for *Pseudomonas* spp. [27]. According to these guidelines, bacterial isolates that differ by a single genetic event, resulting in less than four band differences, are defined as closely related. This may occur within the time-span of an outbreak of about 1–3 months. Isolates with two genetic differences causing four to six band differences in the PFGE profiles are considered possibly related. This may indicate that the isolates are related (especially if the isolates were collected over a longer period, such as 3–6 months), but the isolates may also be possibly unrelated and not involved in the outbreak. Those isolates with ≥7 band differences are different or unrelated [17, 27].

Epidemiological typing studies of *P. aeruginosa* outbreaks using PFGE-*Spe*I indicated that in situations in which the temporal and spatial distribution of isolates does not allow for acquisition of more than three band differences in the PFGE profiles of different isolates, a clonal relatedness and hence transmission can be suggested. Variations of four to six bands seemed to exclude direct transmission, but infection with a clonal variant of the same lineage was considered possible [15, 35].

When using softwares like GelCompar II (Applied Maths) or Fingerprinting Informatix (Bio-Rad) for the analysis of PFGE banding patterns, genetic similarity is often expressed as % similarity by the Dice coefficient. Strains are considered identical if they display 100% similarity, although software-based algorithms in some cases do not match identical isolates at 100%. This indicates that algorithms of mathematical rigidity cannot yet fully replace visual interpretation and analysis of PFGE fragment patterns [36]. The term "PFGE genotype" (genotypes A, B, or C, and so on) is often used to describe isolates with PFGE patterns that fall within an 80% similarity level and consequently form a phylogenetic cluster. Isolates within a genotype not sharing identical patterns are assigned to subtypes (A1, A2, A3, etc). The 80% cutoff value to assign PFGE genotypes was established during studies on *Spe*I macrorestriction patterns to distinguish between geographically and temporally unrelated strains of *P. aeruginosa* [15, 37]. The 80% similarity threshold corresponded to a level above which the majority of sequential isolates from homologous hosts were found, indicating subclonal variation in vivo [33].

Variability of DNA fingerprints among *P. aeruginosa* isolates derived from macrorestriction analysis was suggested to be often the consequence of insertions/deletions rather than single nucleotide polymorphisms (SNPs). During evolution, large fragments of DNA (i.e. genomic islands) may be excised from the *P. aeruginosa* genome or integrated into it. The divergence among *Spe*I PFGE patterns observed among various strains sharing the same SNP profile suggested that the core genome of *P. aeruginosa* is highly conserved and that changes occur at a higher rate in the accessory DNA segments than in the conserved core genome [12].

PFGE was applied as a molecular typing method in a large number of studies to investigate clusters or nosocomial outbreaks of multidrug-resistant *P. aeruginosa*

carrying various resistance determinants such as metallo-β-lactamases (MBLs) or extended-spectrum β-lactamases (ESBLs). A molecular epidemiological examination in 2005 revealed the involvement of hospitals from three different towns in northwest Hungary in an outbreak caused by multidrug-resistant serotype O11 VIM-4 MBL-producing *P. aeruginosa* [38]. PFGE was performed according to the method described by Poh et al. with some modifications [39]. Genomic DNA inserts were digested at 37°C for 2.5 h with 20 U of *Spe*I enzyme. Electrophoresis was performed in a CHEF-DRII apparatus (Bio-Rad). DNA fingerprints were compared by the Fingerprinting II Informatix software (Bio-Rad) using a 1% band position tolerance and a cutoff value of 80% similarity by the Dice coefficient to identify PFGE genotypes. The PFGE-*Spe*I analysis revealed that isolates belonging to the outbreak clone were obtained in a 6 month period from three distinct intensive care units (ICUs) from three different towns and displayed ≥95% similarity by the Dice coefficient. A VIM-4-producing *P. aeruginosa* carrier patient who was transferred between two of the ICUs was also identified providing an epidemiological link between them. A VIM-4-positive isolate from a sink belonged to the outbreak clone, indicating a potential role of a reservoir in the hospital environment in the outbreak besides patient-to-patient transmission [38].

PFGE has proven to be very effective for typing *P. aeruginosa*, providing a high degree of discrimination among strains. However, the sophisticated electrophoresis equipment is available only in specialized laboratories. For *P. aeruginosa*, the most commonly utilized approach for the separation of DNA fragments is contour-clamped homogenous electric field (CHEF)-type apparatus that uses an electrophoresis chamber with multiple electrodes that regularly switch the electric fields at 120° angles during electrophoresis. Moreover, this method is labor-intensive and time-consuming (usually takes 3–7 days), and it is also costly to apply to the study of large numbers of isolates. In addition, results from different laboratories are not readily comparable due to the lack of widely accepted standardized protocols for the typing of *P. aeruginosa* [11, 13, 40].

24.3.3 Amplification-Based Methods

24.3.3.1 Amplified Fragment Length Polymorphisms (AFLP)

AFLP analysis is a selective restriction fragment amplification technique, where adapters are ligated to genomic restriction fragments followed by PCR amplification of these fragments with adapter-specific primers. For AFLP analysis, only a limited amount of purified genomic DNA is needed (50–100 ng); this is digested with two restriction enzymes, one with an average cutting frequency (such as *Eco*RI) and a second one with a higher cutting frequency (such as *Mse*I). After adapter ligation and PCR amplification, polyacrylamide gel electrophoresis of PCR products is performed to obtain a pattern of usually 40–200 bands [41].

The adaptor-specific PCR primers may have an extension of one to three selective nucleotides at their 3′ ends. One selective nucleotide primer amplifies only one out of four of the ligated fragments. For the epidemiological typing of *P. aeruginosa* for general applications, AFLP primers with one selective base (*Mse*I+C) were recommended. The large number of band differences obtained by use of primers without a selective base was considered to be potentially confusing that would usually not give additional information about whether strains are epidemiologically related [25].

Interpretative criteria based on visual inspection of AFLP banding patterns of *P. aeruginosa* isolates were developed by Spejer and colleagues. The criteria for AFLP analysis with *Eco*RI and *Mse*I restriction enzymes and primers with one selective base were that isolates with less than three band differences by AFLP analysis were considered as "probably related." *P. aeruginosa* strains displaying three to four band differences by AFLP analysis were classified as "possibly related," in analogy with four to six band differences by PFGE typing. In this work, based on computer-assisted evaluation of the obtained AFLP data, the cutoff value for strain differentiation was set as 80%. Some AFLP patterns that were read as identical by visual examination were found to display 85–98% similarity by software. Thus, it was recommended that visual inspection of clusters generated by the software is always necessary and that the epidemiological data should also be taken into account when analyzing whether genetically similar strains are also epidemiologically related [25].

By use of fluorescent primers and analysis of fluorescently labeled PCR products on an automatic sequencer, AFLP may be performed within 24 h [42]. The profiles generated with labeled primers and an automated sequencer are highly complex, and the use of software for cluster analysis is therefore highly recommended. The data files from the sequencer, such as ABI PRISM 310 (Applied Biosystems) or Vistra 725 DNA sequencer (Amersham), can be transferred to a software, like Molecular Analyst Fingerprinting (Bio-Rad) or GelCompar software (Applied Maths). Following conversion and normalization of the data, levels of similarity between fingerprints can be calculated with the Pearson product moment correlation coefficient [41]. The Pearson correlation coefficient was recommended for AFLP analysis as it is independent of the relative intensities of patterns and also does not suffer from subjective band detection and band-matching criteria by comparing the entire profile as opposed to the detection of specific bands [16]. However, an automated sequencer is less likely to be available for the analysis of fluorescently labeled PCR products for most routine clinical laboratories.

A comparative study of the PFGE and AFLP methods with 22 *P. aeruginosa* isolates revealed 100% typeability for AFLP and a DI of 0.97 as opposed to 0.96 for PFGE. In this study, the *Eco*RI and *Mse*I restriction enzymes were used for AFLP and clusters of epidemiologically related *P. aeruginosa* isolates having indistinguishable PFGE patterns displayed >90% homology by AFLP. On the other hand, epidemiologically unrelated strains with more than six band differences by PFGE displayed <90% homology by AFLP using a GelCompar software-assisted analysis of fluorescent-labeled AFLP fingerprints [43].

The AFLP method was fast enough to allow a "real-time" monitoring of an outbreak of *P. aeruginosa* infections in the Careggi hospital (Florence, Italy), permitting additional preventive measures and suggested an environmental source of infection. AFLP patterns of the same *P. aeruginosa* strains were consistent over time; however, occasionally differences of one or two bands were observed between the AFLP patterns of multiple isolates of the same strain [44]. These differences result in an about 87% reproducibility of the marker pattern for the same isolates [45].

Commercial kits are also available for the AFLP typing of *P. aeruginosa*, such as the AFLP Microbial Fingerprinting kit (Applied Biosystems). The hands-on time of AFLP analysis is between those of RAPD analysis and PFGE (in the order of RAPD < AFLP < PFGE) [25]. Although AFLP typing was useful in investigating the molecular epidemiology of multidrug-resistant *P. aeruginosa* colonization in a Burn Unit of the Queen Astrid Military Hospital, Belgium, the authors recommended the use of RAPD-PCR for genotyping of surveillance isolates, which is less expensive and time-consuming than AFLP to confirm an outbreak [46].

24.3.3.2 Random Amplification of Polymorphic DNA (RAPD)

During RAPD, one PCR primer with usually about 10–15 bases is used, which is not targeted to amplify any specific bacterial DNA sequence. In most protocols, at least in the first few cycles of PCR, low annealing temperatures are used that allows imperfect hybridization at multiple random locations on the chromosome to amplify random sections of it. The PCR products are separated by agarose gel electrophoresis, stained, and analyzed by visual inspection or by computational methods. This method was found simpler, faster, and less costly and labor intensive than PFGE, and several other molecular typing techniques by many laboratories.

The RAPD typing approach of *P. aeruginosa* clinical isolates using 10-nucleotide primers was evaluated on a total of 200 isolates and yielded 100% typeability and 98.5% intralaboratory reproducibility [47]. However, the banding patterns observed for the same *P. aeruginosa* isolates typed by the same RAPD method in the different laboratories can show substantial differences. It was reported that most aspects of the PCR procedure, including small differences in the temperature profile and in the source and different batches of the Taq polymerase, may affect the reproducibility of the banding patterns. RAPD banding patterns are most reproducible if the assay is performed repetitively under identical conditions, equipment, and the same operator, and if it is run on a regular rather than a sporadic basis. Thus, RAPD results are not readily transportable and, as such, can be used only for intralaboratory comparison of *P. aeruginosa* strain collections [48].

On the other hand, comparison of the dendroGrams obtained in three different laboratories revealed an overall good reproducibility of RAPD in identifying different clusters of *P. aeruginosa* isolates. RAPD could reproducibly assign the *P. aeruginosa* isolates to different clusters that also corresponded to the clonal complexes identified by multilocus sequence typing (MLST) (see Sect. 3.4.1) [48]. The RAPD

method also allowed the identification of RAPD clusters that correlated strongly with multiple locus enzyme electrophoresis *P. aeruginosa* populations. These concordant observations suggest that the RAPD method is valid for the detection of genetic links between various distinct populations of *P. aeruginosa* isolates [49].

During epidemiological studies at the National Center for Epidemiology, Budapest, 10–25 bands of 200–3,000 bp for the individual *P. aeruginosa* isolates were obtained when applying the method evaluated by Campbell and colleagues [47, 50] by use of the 10-nucleotide primer 208, an Invitrogen Taq polymerase and 40 ng purifed genomic DNA. Variations in the concentration of genomic DNA did not have a significant effect on the obtained patterns; however, the type of Taq polymerase used had a profound impact on the number and intensity of the PCR products. Best results (i.e. highest number and intensity of bands) were gained with the Invitrogen Taq polymerase [48, 51, 52].

Observation made during comparative studies of various molecular typing methods of *P. aeruginosa* revealed different resolution powers for the different typing methodologies, in the following order: PFGE = AFLP > RAPD > MLST [25, 48]. Interpretative criteria for RAPD banding patterns are less well defined than those established for PFGE because the interpretation of band differences remains as yet biologically unfounded [16]. In one study, a cutoff value for *P. aeruginosa* strain differentiation was set at 90% similarity of RAPD banding patterns [25]. Campbell and colleagues using 10-nucleotide primers found that epidemiologically related *P. aeruginosa* isolates, such as multiple isolates from the same patients, could be read as identical in their RAPD patterns. *P. aeruginosa* isolates with no more than one major band or three minor band differences were defined as "possibly related" and subjected to further analysis by PFGE. Isolates that were epidemiologically unrelated each had a unique RAPD pattern [47].

RAPD typing of MDR *P. aeruginosa* clinical isolates from Hungary and other European countries using software-assisted evaluation indicated that epidemiologically related *P. aeruginosa* isolates (such as isolates belonging to the same epidemic clone spreading in a hospital) displayed genetic similarities >90% (data not shown). Isolates sharing ≥80% similarity belonged to the same clonal complex as determined by MLST, although some isolates of the same MLST-complex clustered together by RAPD but with genetic similarities lower than 80% [48, 51, 52].

Taken together, several molecular epidemiological investigations indicate that RAPD could serve as a first screen for the epidemiological typing of *P. aeruginosa* because of the simplicity and high speed of this technique and because the bacterial grouping results attained are in good correlation with macrorestriction analysis and MLST [35, 48, 51]. Through screening by RAPD, clonal relatedness can be determined in about 24 h and with a relatively low cost. This would enable clinical microbiologists to unravel most of the nosocomial epidemics. In a second step, PFGE can be used if necessary for the confirmation of the RAPD results and for fine-tuning the infection control or clinical measures already initiated on the basis of the RAPD data [35].

24.3.3.3 Multilocus Variable-Number Tandem Repeat (VNTR) Analysis (MLVA)

Many bacterial genomes contain short, repetitive tandem sequences. The copy number of these VNTR sequences often varies between unrelated strains and therefore can be used for genotyping. VNTR typing utilizes PCR to amplify the whole repeat region. Following amplification, the PCR products are separated by electrophoresis, and their size is determined to identify the number of repeats present at the locus under investigation. In most cases, multiple loci (repeat regions or VNTRs) are analyzed to determine the genotype. Each strain is described by a code corresponding to the number of repeats at the selected VNTRs. These codes can be easily compared between different laboratories through Web-based databases, such as the one for *P. aeruginosa* available at http://bacterial-genotyping.igmors.u-psud.fr [53].

The MLVA scheme was developed for *P. aeruginosa* by Vergnaud and colleagues and was subsequently improved by the addition of new epidemiologically informative markers [53, 54]. This MLVA scheme involves 15 loci with repetitive tandem sequences (VNTRs). The MLVA genotype of a *P. aeruginosa* isolate with 15 VNTRs (MLVA15) is expressed as its allelic profile showing the number of repeats at each analyzed VNTR. A new genotype number is given when one difference is observed at any VNTR out of the 15. Lineages are defined as groups of isolates for which the genotype differs at a maximum of two loci (VNTRs). The global index of diversity for the 15 markers calculated with 190 isolates was 0.97. For an easier and more robust MLVA scheme, the number of characterized VNTRs can be reduced to 10 when only minisatellites ms142, ms211, ms212, ms213, ms214, ms215, ms216, ms217, ms222, and ms223 are involved in the analysis [53].

The MLVA genotypes of the *P. aeruginosa* strains recovered from individual patients over time proved stable, except for the occasional insertion of an IS element and the addition or deletion of repeats in a single VNTR. MLVA was used to survey the sources of *P. aeruginosa* infections in a pediatric CF center in Paris, France. Between January 2004 and December 2006, *P. aeruginosa* was detected in 46 children, 17 of whom had primary colonization. A total of 163 isolates were recovered. Upon genotyping with 15 VNTRs, a total of 39 lineages composed of indistinguishable or closely related isolates were observed. One of them corresponds to "clone C," which is widely distributed in Europe [53].

Using six of the loci in a study analyzing a total of 81 non-repeat clinical *P. aeruginosa* isolates collected at two university and two regional hospitals during 2004–2008 in Bulgaria, 100% reproducibility and 97.5% typeability were determined for this MLVA scheme [31]. To investigate the different features of MLVA and MLST (see Sect. 3.4.1) in comparison with PFGE for genotyping *of P. aeruginosa*, 32 strains derived from sputum samples of Dutch CF patients were typed with each three methods. Only 9 of the original 15 VNTRs were analyzed. PFGE, MLVA, and MLST had DIs of 0.988, 0.980, and 0.952, respectively, with overlapping 95% confidence intervals. A high congruence at the level of clonal clusters was observed between the three methods. The authors underscored the advantages of MLVA and

MLST in their portability and ease of interpretation, and a further advantage of MLVA over MLST was also highlighted in being more cost effective as it does not require sequencing [55].

A potential drawback of the MLVA method is that the accurate sizing of fragments, even using fluorescent detection systems, may not be simple, as it is mobility dependent and depends on the sequence composition as well. Furthermore, the evolution of repetitive DNA sequences may be too rapid, compromising epidemiological concordance. Although to date few epidemiological studies were performed using this method, MLVA may become a more widely used typing technique for *P. aeruginosa* in the future, especially in regional or in international studies where inter-laboratory or intercontinental comparisons of portable typing data are required [16, 55].

24.3.4 Sequencing-Based Method

24.3.4.1 Multilocus Sequence Typing

MLST is one of the most successful techniques developed for the molecular typing of bacteria that has been used in an increasing number of studies in the past few years also for *P. aeruginosa*. An MLST protocol that is also connected to a public database (www.pubmlst.org/paeruginosa) was developed by Dowson and colleagues [26]. During MLST, the integral fragments of seven housekeeping genes are amplified and sequenced on both DNA strands, and the obtained sequences are searched against the internet-based MLST database for assignments of allelic numbers. Each different allelic combination (or allelic profile) is assigned a sequence type (ST) using the MLST database.

The seven genes selected for use with the MLST scheme for *P. aeruginosa* were *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*. Criteria governing the selection of the loci included their biological role (diverse housekeeping roles like mismatch repair, DNA replication, and amino acid biosynthesis), the necessary length of DNA sequence (about 600 bp), and the chromosomal location of the genes (i.e., a minimum of 6 kbp upstream or downstream from known virulence factors, lysogenic phages, or insertion sequence elements). Moreover, factors such as suitability for nested PCR primer design and an appropriate level of sequence diversity had to be also considered [26].

During PCR amplification of the target sequences, some modifications of the PCR procedure may be necessary for certain *P. aeruginosa* isolates. These modifications include the addition of pure dimethyl sulfoxide at a concentration of 5 µl per 100 µl PCR master mix and increasing the annealing temperature from 55 to 58°C [48]. Also, for generating clear and unambiguous DNA sequence data for searches against the MLST database in some cases, it is helpful to perform additional sequencing reactions on the purified PCR fragments also with the amplification primers.

Isolates with the same ST are considered as members of the same clone. Clonal complexes may be defined as *P. aeruginosa* isolates sharing ≥5 identical alleles out of the 7 analyzed loci [26] or according to a more stringent general definition as sharing ≥6 identical alleles [56]. The eBURST software can be used for phylogenetic analysis and identification of clonal complexes as described by Feil and colleagues ([56]; http://eburst.mlst.net/).

In a comparative typing study of 90 *P. aeruginosa* isolates obtained from cultures of perirectal surveillance swabs from patients in an intensive care unit, PFGE was found to have a greater discriminatory power than MLST, with DI values of 0.999 compared to 0.975, respectively. Thus, both of these two methods displayed high discriminatory abilities, but PFGE distinguished more types (85 versus 60 types distinguished by MLST) [57]. Further molecular typing studies indicated that MLST was a key epidemiological tool for studies of regional and global epidemiology of multidrug-resistant *P. aeruginosa* because the results are highly reproducible and readily portable between different laboratories, the interpretation of data is unambiguous, and moreover, it is particularly useful for determining clonal relationships and genetic diversity among bacterial strains with great differences in their temporal and geographical origin. These works underscored the importance of two clonal complexes comprising mainly serotype O11 and O12 isolates, respectively, in the dissemination of bla_{VIM} MBL and $bla_{\text{PER-1}}$ ESBL harboring *P. aeruginosa* strains in Europe [48, 51, 56, 58].

Between 2003 and 2008, a number of molecular typing studies were performed at the National Center for Epidemiology, Budapest, Hungary, to identify major multidrug-resistant nosocomial clones of *P. aeruginosa* responsible for the emergence and spread of acquired antibiotic resistance genes in the health-care institutions. A collection of altogether about 1,500 *Pseudomonas* spp. clinical isolates were screened for MBL and ESBL production, and selected isolates also for the overexpression of AmpC β-lactamase and the presence of integron-borne aminoglycoside resistance determinants. Representative MDR *P. aeruginosa* isolates were subjected to a variety of typing methods including serotyping, RAPD, and MLST. These studies revealed the pivotal role of four distinct *P. aeruginosa* clonal complexes (as determined by MLST) in the emergence of MDR isolates. The first of these clonal complexes (CC4) is characterized by serotype O12, the founder sequence type ST111, and corresponds to the major European multiresistant P12 clone. Certain isolates of this clonal complex in Hungary are involved in the dissemination of VIM-4 MBLs. The second complex (CC11) is characterized by serotype O11, the founder sequence type ST235, and contributes to the spread of VIM-4 MBLs and PER-1 ESBLs. The remaining two clonal complexes with a countrywide distribution are characterized by serotypes O4 and O6 and sequence types ST175 and ST395, respectively, and contain isolates that overproduce the chromosomal AmpC β-lactamase and also carry integron-borne *aadB* (aminoglycoside 2′-*O*-adenylyltransferase) genes. All these four clonal complexes appear to have a wide geographical distribution also outside Hungary, but the acquired resistance determinants may display high variability between isolates of distinct geographical origins [38, 51, 52, 58].

Based on the observed variability of the resistance genes carried by complexes CC4 and CC11 in different countries and hospitals, the concept of multiple independent acquisitions of these resistance determinants was proposed by members of these two widespread clonal lineages of *P. aeruginosa* that seem to be particularly adept at acquiring resistance determinants that result in an MDR phenotype [48, 51, 59, 60].

In summary, it could be concluded that by applying commonly used cutoff values, PFGE and RAPD were unable to demonstrate clonal relationships between some *P. aeruginosa* isolates that belonged to the same ST or clonal complex by MLST. These observations highlight the additional information that MLST can provide in molecular typing of bacterial infections [48]. MLST is the current method of choice for performing regional and international epidemiological typing studies, and the one with the soundest biological basis. Although MLST generates unambiguous, reproducible, and transportable isolate profiles, due to the expense, labor, and time involved in the analysis, it is not the method of choice for routine epidemiological typing of a large number of isolates during investigations of local outbreaks.

24.3.5 Molecular Typing of Pseudomonas spp. Other Than P. aeruginosa

This section briefly summarizes the typing methods available and most widely used for *Pseudomonas* spp. clinical isolates other than *P. aeruginosa*, such as *P. stutzeri, P. putida,* and *P. fluorescens* that have substantially lower prevalence rates among clinical isolates than *P. aeruginosa* (see Sect. 1). The general interpretative rules and features of molecular typing methods for *Pseudomonas* spp. isolates may be assessed based on the sections above discussing those for *P. aeruginosa*, the type species of the genus.

Similar to *P. aeruginosa*, PFGE using the *Spe*I enzyme may be considered as the gold-standard typing tool for all these species that is also often used to investigate outbreaks and clusters of various types of infections caused by these microbes. Analysis by PFGE-*Spe*I was found to be an accurate and reproducible method for the typing of *P. fluorescens*, *P. putida,* and *P. stutzeri* isolates, where the commonly applied criteria developed by Tenover and colleagues were used for interpretation of banding patterns and an 80% cutoff value for establishing clonal relatedness by computer-assisted analysis of PFGE gels [61–63]. In some studies, amplification-based techniques such as AFLP were used for the characterization of various strains of *Pseudomonas* spp. [64, 65].

MLST schemes with internet-based public MLST databases are not yet available for these species; however, combined phylogenetic and multilocus DNA sequence analyses (MLST) were applied in some studies on *P. stutzeri* and other *Pseudomonas* spp. isolates of various origin [66, 67]. With the absence of publicly available MLST databases and standardized MLST protocols for these bacteria, MLST typing of

Pseudomonas spp. isolates other than *P. aeruginosa* may presently remain in the scope of fundamental research activities rather than a widely used tool for epidemiological investigations.

24.4 Conclusions

When *Pseudomonas* spp. isolates are recovered from normally sterile sources (such as blood or cerebrospinal fluid) or from multiple patients of a hospital department, an epidemiological investigation should be initiated. Isolates from the concerned patients should be examined for clonal relatedness by a molecular typing technique, such as RAPD and/or PFGE. If a clonal cluster is detected, the source of infections should be identified and a strategy should be defined for infection control and decontamination. Since the use of molecular typing usually involves significant costs and hands-on time, there should always be a clear objective for its application and a strategy to optimize these expenditures [11, 13].

For *P. aeruginosa* isolates, an initial screening may be performed by RAPD typing to determine clonal relatedness at a high speed and a relatively low cost. This step could unravel most of the nosocomial epidemics. In a second stage, PFGE may be used if necessary for confirmation of the RAPD results and for fine-tuning the infection control or clinical measures already initiated on the basis of the RAPD data [35]. In general, repetitive typing or use of a second method does not significantly alter the epidemiologic assessment if the isolates are typeable and appropriate controls (isolates from epidemiologically related and unrelated patients) are used in the analysis.

The use of RAPD as a first screening method for the local epidemiological investigations of *P. aeruginosa* nosocomial strains would also be supported by its technical simplicity once the technique has been set up in the laboratory. Compared to, for example, MLVA, there is a need to set up only 1 PCR reaction and to use only 1 lane during agarose gel electrophoresis per isolate compared to 7–15 PCR reactions and lanes per isolate when using MLVA.

Although PFGE-SpeI remains the gold standard or reference method for the molecular epidemiological typing of *Pseudomonas* spp., this technique is not the method of choice for studies of large populations of strains collected over extended periods of 1 year or longer and/or from diverse geographical locations. While evaluating PFGE banding patterns, differences in levels of genetic similarity between some *P. aeruginosa* isolates may be observed as determined by visual inspection vs. computational methods. Similar findings were reported by several publications together with recommendations that there is a need for an effective standardization and development of the software packages and applications used for the analysis of PFGE patterns. These observations and recommendations underscore the advantages of the MLST method in providing unambiguous interpretation of experimental data and in establishing clonal relatedness between isolates when this is not readily apparent using PFGE [27, 36, 48, 58]. A proposed scheme summarizing

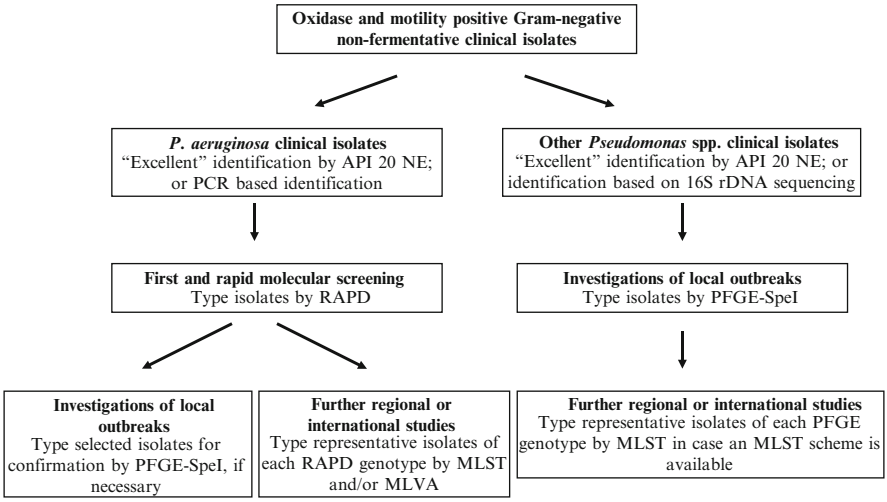
Oxidase and motility positive Gram-negative non-fermentative clinical isolates
P. aeruginosa clinical isolates
"Excellent" identification by API 20 NE; or PCR based identification
Other Pseudomonas spp. clinical isolates
"Excellent" identification by API 20 NE; or identification based on 16S rDNA sequencing
First and rapid molecular screening
Type isolates by RAPD
Investigations of local outbreaks
Type isolates by PFGE-SpeI
Investigations of local outbreaks
Type selected isolates for confirmation by PFGE-SpeI, if necessary
Further regional or international studies
Type representative isolates of each RAPD genotype by MLST and/or MLVA
Further regional or international studies
Type representative isolates of each PFGE genotype by MLST in case an MLST scheme is available

Fig. 24.1 A proposed tentative scheme for the molecular typing of *Pseudomonas* spp. clinical isolates. Please see text for further details and references

these findings is presented in Fig. 24.1 that may serve as an additional guide when planning local and/or larger-scale epidemiological studies on clinical isolates of the *Pseudomonas* genus.

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Part VII
Emerging and Re-emerging Pathogens

Chapter 25
Acinetobacter baumannii

Lenie Dijkshoorn

25.1 Introduction

Bacteria of the genus *Acinetobacter* are widespread in nature. They can be found in soil and water and in samples from humans and animals. The organisms have long been considered harmless for humans, but this view changed by the 1970s when it was noted that epidemic, multidrug resistant (MDR) strains were increasingly isolated from severely ill patients [1]. Since then, the taxonomy has been refined and methods have been developed to identify clinically important strains and species. Reviews of the clinical importance and biology of these organisms have been given [2–4]. The present chapter deals with the complexity of the genus and, then, focuses on clinically relevant species. State-of-the-art-methods for characterization of these organisms will be presented.

25.2 Microbiology of Acinetobacters

The microbiology and physiology of acinetobacters have been reviewed by Juni [5]. Bacteria of the genus *Acinetobacte*r are non-motile, coccoid, or rod-shaped organisms. In Gram staining, they are Gram-negative, or slightly Gram-positive. They are non-motile, oxidase-negative (Kovács technique), catalase-positive, non-fermenting, and most strains do not reduce nitrate in the conventional nitrate reduction assay. Depending on the species, glucose can be acidified aerobically and haemolysis of sheep blood can occur. Most organisms grow between 20 and 37°C, while *Acinetobacter baumannii* and *A. nosocomialis* even grow at 44°C [6]. Bacteria

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belonging to the genus *Acinetobacter* have the ability to transform the mutant strain *Acinetobacter baylyi* BD413 from auxotrophy to prototrophy, a feature that can be used for genus identification [7].

25.3 Taxonomy of the Genus *Acinetobacter*

Acinetobacters belong to the Gammaproteobacteria. The history of the genus *Acinetobacter* begins in the early 1900s with the description of an organism, *Micrococcus calco-aceticus*, which was recovered from soil by enrichment cultivation with calcium acetate as a single carbon source [8]. Later, similar organisms were described independently and labelled by different names. A comprehensive study of these organisms, at the time classified as the "oxidase-negative *Moraxella* group," revealed a considerable nutritional variability [9]. However, no clear-cut phenotypic criteria for the delineation of species were found at the time and, therefore, the species were lumped together into one genus, *Acinetobacter*, comprising only one species, *Acinetobacter calco-aceticus* [10].

A milestone in the development of the taxonomy of the genus *Acinetobacter* was the description of 12 DNA-DNA hybridization groups in 1986, six with valid species names (Table 25.1) [11]. Since then, additional DNA-DNA hybridization groups and species have been described. To date, 27 validly named species (http://www.bacterio.cict.fr) and nine DNA-DNA hybridization groups with provisional designations are known (Table 25.1). An important recent nomenclatural proposal was the assignment of the names *Acinetobacter pittii* and *Acinetobacter nosocomialis*, to *Acinetobacter* genomic species 3 and 13TU, respectively [6] (Validation List 140 of IJSEM July 2011). *A. baumannii*, *A. pittii, A. nosocomialis*, and the environmental species *A. calcoaceticus* are genotypically closely related and phenotypically difficult to distinguish and are, therefore, as a group referred to as the *A. baumannii-A. calcoaceticus* (Acb) complex [12].

Despite the progress made to subdivide the genus, the taxonomy remains complex. For example, the provisional designation of a number of DNA-DNA hybridization groups (DDH groups, genomic species, gen. sp.) is confusing. Identical designations, i.e., 13–15, have been given independently to three genomic species in two reports [13, 14] (Table 25.1). To differentiate between these taxa, the addenda TJ or BJ are added commonly to the respective genomic species names. Furthermore, it has been shown that several names, e.g., gen. sp. 13BJ and 14TU, and genospecies 9 [11] and *A. lwoffii* are likely synonyms for the same species (Table 25.1) [14]; *Acinetobacter grimontii* is a junior synonym of *Acinetobacter junii* [15]. Several named and yet unnamed species have been described on the basis of one or a few strains only, which is common practice in today's taxonomy although disputed by some taxonomists. The variation of these species is not known, which makes it difficult to identify isolates to these species.

Table 25.1 Classification of the genus *Acinetobacter*

Species with valid names	Cultured from*	References
A. calcoaceticus	Soil, human (incl. clinical specimens)	[6, 11, 14]
A. baumannii	Human and animals (incl. clinical specimens)	[6, 11, 14]
A. pittii (genomic species 3)	Human (incl. clinical specimens)	[6]
A. nosocomialis (genomic species 13TU)	Human (incl. clinical specimens)	[6]
A. haemolyticus	Human (incl. clinical specimens)	[11, 14]
A. junii	Human (incl. clinical specimens)	[11, 14, 15]
A. johnsonii	Human (incl. clinical specimens), animals	[11, 14]
A. lwoffii (incl. genomic sp. 9)	Human (incl. clinical specimens), animals	[11, 14]
A. radioresistens	Human (incl. clinical specimens), soil, cotton	[11, 14, 102]
A. ursingii	Human (incl. clinical specimens)	[103]
A. schindleri	Human (incl. clinical specimens)	[103]
A. parvus	Human (incl. clinical specimens), animals	[104]
A. baylyi	Activated sludge, soil	[105]
A. bouvetii	Activated sludge	[105]
A. towneri	Activated sludge	[105]
A. tandoi	Activated sludge	[105]
A. grimontii	Activated sludge	[105]
A. tjernbergiae	Activated sludge	[105]
A. gerneri	Activated sludge	[105]
A. beijerinckii	Human (incl. clinical specimens), animals	[58]
A. gyllenbergii	Human (incl. clinical specimens)	[58]
A. bereziniae (genomic sp. 10)	Human (incl. clinical specimens), animals, environmental specimens	[11, 14, 53]
A. guillouiae (genomic sp. 11)	Human (incl. clinical specimens), environmental specimens	[11, 14, 53]
A. soli	Soil	[106]
A. venetianus	Sea water, lake water, vegetables	[64]
A. brisouii	peat	[108]
A. rudis	milk, waste water	[109]

Species with provisional designations**	Cultured from*	References
Genomic sp. 6	Human (incl. clinical specimens)	[11, 14]
Genomic sp. 13BJ/14TU	Human (incl. clinical specimens)	[13, 14]
Genomic sp. 14BJ	Human (incl. clinical specimens)	[13]
Genomic sp. 15BJ	Human (incl. clinical specimens)	[13]
Genomic sp. 16	Human (incl. clinical specimens)	[13]
Genomic sp. 17	Human (incl. clinical specimens)	[13]
Genomic sp. 15TU	Human (incl. clinical specimens)	[14]
Genomic sp. “Between 1 and 3”	Human (clinical specimens)	[107]
Genomic sp. “Close to 13TU”	Human (clinical specimens)	[107]

* Culture results are those from the references describing the species and may not cover the true ecology of the species

** Delineated by DNA-DNA hybridization

25.4 The Epidemiology of Acinetobacters with Emphasis on *A. baumannii*

The knowledge of the ecology of most *Acinetobacter* species is limited. Many of the species listed in Table 25.1 have been found in human clinical specimens but only a few are known to be clinically relevant. Even those with clinical relevance are opportunistic pathogens that usually only affect severely ill, hospitalized patients. An eight years' study in a university hospital using validated identification methods identified *A. baumannii* and *A. pittii* (27% and 26%, respectively) as the most important species [16]. The closely related species *A. pittii* and *A. nosocomialis* may play a role similar to *A. baumannii* in hospitals, but the relative distribution of these three Acb complex species both in hospitals and their occurrence in the community and in environmental specimens seem to vary geographically [4, 17, 18].

Apart from *A. baumannii* and its closely related species *A. pittii* and *A. nosocomialis*, several other species have been associated with hospital acquired infections. These include species like *A. lwoffii*, *A. johnsonii*, *A. junii*, *A. haemolyticus*, *A. parvus*, *A. ursingii*, and *A. schindleri*, some of which may occur normally on the skin [4]. Infections with these organisms are generally less common that those with *A. baumannii* and are frequently iatrogenic of origin.

Studies from before 1986 when only one species (*A. calcoaceticus*) was known have reported recovery of acinetobacters from soil and water [19]. This may have led to the assumption that *A. baumannii* also has its reservoir in the soil [20], but evidence for this is limited. *A. baumannii* was found to be infrequent on the skin of healthy individuals [21]. In Hong Kong, relatively high skin carrier rates have been noted for *A. pittii* [22]. This species was also found on local vegetables and in the soil, as was the case for *A. baumannii* although less frequent than *A. pittii*. In colonized or infected patients, acinetobacters (most of them identified to *A. baumannii*; Dijkshoorn, unpublished results) can be found on the skin and mucous membranes [23]. In hospitals, *A. baumannii* has not only been found in patients but also in their vicinity [24]. It is assumed that the colonized patient is generally the source from which the organism spreads to the local environment and to other patients, for example by air, hands of staff, or contaminated equipment [4, 25, 26]. The ability of the organisms to resist chemical agents and desiccation is considered an important attribute to survive in the hospital environment. A recent study in a German veterinary clinic has shown the emergence of *A. baumannii* in hospitalized small animals such as cats and dogs that were mainly located on the intensive care unit (ICU) [27]. It remains to be assessed whether these organisms spread from humans to animals or the other way round, or whether these organisms constitute a group specifically associated with these animals.

25.5 The Clinical Significance of *A. baumannii*

A. baumannii can give rise to severe infections including pneumonia, blood stream infections, meningitis, and wound and urinary tract infections, but colonization is

generally more common than infection [3, 4]. Differentiation between these two states can be difficult in critically ill patients. Morbidity and mortality rates differ considerably in the literature, and there is a continuous debate on the question to what extent the organism contributes to mortality [28].

The main patient at risk for acquisition *A. baumannii* is the severely ill patient located in intensive care units, burn wards, and sometimes on neonatal care units. In recent years a special category of patients has emerged, i.e., severe trauma patients wounded during war actions [29, 30] or natural disasters [31] that are particularly vulnerable to MDR *A. baumannii*.

A major problem of *A. baumannii* is the fact that MDR strains of this species have the ability to spread among patients and to persist in the hospital environment and their eradication is difficult.

25.6 Antibiotic Resistance

A. baumannii ranks high among the so-called alert organisms, bacteria that are notorious for their association with healthcare-associated infections and their resistance to multiple antibiotics. An increasing proportion of *A. baumannii* is resistant to carbapenems, which have long been considered the last resort for treatment. Thus, only a few options for treatment remain including colistin, tigecycline, sometimes sulbactam (given in combination with ampicillin) or combinations of other antibiotics [4]. Colistin has the reputation to be toxic, but if carefully administered, this may be an option.

All known mechanisms leading to antibiotic resistance, including decreased influx, enzymatic degradation, altered targets, and active efflux have been found in *A. baumannii*. The genes associated with resistance can be chromosomal or located on plasmids. The mechanisms and epidemiology of carbapenem resistance of *A. baumannii* have been reviewed by Poirel and Nordmann [32]. Resistance to carbapenem is frequently caused by class D carbapenem hydrolysing beta-lactamases, which are clavulanic acid-resistant and belong to three unrelated groups represented by OXA-23, OXA-24, and OXA-58 [32], and the recently discovered OXA-143 [33], the genes of which can be on plasmids or chromosomal. One intrinsic carbapenem hydrolyzing oxacillinase, OXA-51, has variable expression depending on the presence of an insertion sequence, IS*AbaI*, located upstream of the OXA-gene [34]. Carbapanem resistance may also be associated with porins or penicillin-binding proteins. In epidemiological studies of carbapenem resistant studies, it is increasing practice to assess the OXA-genes of the strains involved [35].

Antibiotic resistance in non-*baumannii* species is generally low, but appearance of resistance to multiple antibiotics including carbapenems in *A. pittii* (gen. sp. 3) and *A. nosocomialis* (gen. sp. 13TU) is worrying [17]. Detection of the $bla_{\text{OXA-23 like-}}$oxacillinase gene in *Acinetobacter radioresistens* has given rise to the presumption that this species might serve as a source for this gene [36]. It is noteworthy that, despite the increasing occurrence of MDR *A. baumannii* in hospitals, substantial numbers of isolates of this species were found to be susceptible to antibiotics [16, 37].

25.7 Diversity Within *A. baumannii*

Typing studies using various methods have shown a great diversity within *A. baumannii*. Nevertheless, three groups of highly similar strains were distinguished among isolates from different hospitals in NW Europe as revealed by a combination of typing methods [38, 39]. It was suggested that these groups represent clones (European (EU) clones I, II, and III) sensu Ørskov and Ørskov [40] i.e., groups of isolates without a direct epidemiological link but so similar that they are assumed to be descendants of a common ancestor. Many papers have meanwhile indicated that EU clones I and II are responsible for outbreaks worldwide. Additional clones have been reported [30, 41, 42]. Originally, clones were delineated by a polyphasic approach using a combination of methods including cell envelope protein analysis, ribotyping, and AFLP analysis [38, 43]. More recently, multilocus sequence typing (MLST) has appeared as a universal approach to identify acinetobacters to these and to tentative clones [44]. A multiplex PCR targeting three genes for rapid identification of the three EU clones has also been described [45].

25.8 Sequenced Genomes

Twenty-six *Acinetobacter* genomes are listed on the NCBI site (http://www.ncbi.nlm.nih.gov/sites/entrez) as of 24 May 2011. These include 14 *A. baumannii* strains and 12 strains of other species. A number of these, including the type strain of *A. baumannii* (ATCC 19606^T), *A. pittii* (SH024), *A. nosocomialis* (RUH2624), and *A. calcoaceticus* (RUH2202) have been sequenced as part of the Human Microbiome Project ("*Acinetobacter* group Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org)").

The genome sizes are in the range of 3.1–4.0 Mb. Genomic studies have revealed occurrence of multiple genomic islands with clustered groups of genes (including pathogenicity or resistance genes) in *A. baumannii*. Up to 36 putative alien islands were found in the MDR, epidemic strain ACICU [46]. A particular group, the *A. baumannii* resistance islands (AbaRs), comprises variable numbers of genes encoding proteins related to antibiotic resistance and is inserted at the same position of a chromosomal ATPase gene [47]. A study of 26 MDR *A. baumannii* strains of EU clone I obtained from 1984 to 2005 revealed that all strains had the same structure (AbaR3) or a derivative of it [48]. It was postulated that this structure has conferred EU clone I with a selective advantage to spread over European hospitals.

25.9 Methods for *Acinetobacter* Species Identification

Species identification with commercial identification systems based on phenotypic characters as done in diagnostic microbiology is not feasible for most *Acinetobacter*

species. These systems are generally adequate for genus identification or identification to the Acb complex, which may suffice in individual patient cases if combined with antibiotic susceptibility. However, precise species identification is important to study the ecology and biological significance of species and their possible clinical-epidemiological role. An overview of methods for *Acinetobacter* species identification is given in Table 25.2, and selected methods are discussed in the following paragraphs.

25.9.1 Phenotypic Characterization

25.9.1.1 Biochemical and Physiological Profiling

A phenotypic identification system comprising more than 20 physiological, nutritional, and enzymatic tests has been developed in the 1980s to identify the described (genomic) species [11]. This system has appeared instrumental in the delineation and characterization of additional *Acinetobacter* species, although not all species, like the four species in the Acb complex and several haemolytic species, can be differentiated well [12, 13]. Nevertheless, the system or selected tests can be useful in combination with amplified 16S ribosomal DNA restriction analysis (ARDRA) ("consensus identification") [49]. The phenotypic system is not commercially available and not widely used. Previous studies have indicated that commercial identification systems perform insufficiently for *Acinetobacter* species identification [50], but there is no recent systematic evaluation of these systems and their databases in light of the current taxonomy. The difficulties for species identification have triggered the development of a variety of genotypic methods (Table 25.2).

25.9.1.2 Methods Based on Chemotaxonomic Markers

Biochemical compounds including macromolecules like proteins, fatty acids, liopopolysaccharides, or enzymes are considered useful taxonomic markers, depending on the genus or species. Investigation of the cellular fatty acid composition for *Acinetobacter* species identification for the then known 18 (genomic) species was not very promising as only two main groups of species could be differentiated [51]. Cell-envelope protein profiling using sodiumdodecyl sulphate-polyacrylamide gel elctrophoresis (SDS-PAGE) has shown that the profiles comprising heavy and less dense protein bands could be used for *Acinetobacter* species identification [52]. However, this approach became obsolete with the introduction of DNA-based methods. Currently, matrix-associated laser desorption/ionization time-of-flight (MALDI TOF)—mass spectrometry (MS) is revolutionizing microbial diagnostics. A widely used, commercial system separates proteins and peptides by their mass, which results in a characteristic peak pattern. As might be expected from protein profiling, provisional MS results have shown promising results to differentiate some

Table 25.2 Genotypic methods for *Acinetobacter* species, clone, and strain identification

Method	Target structure	Application*	Reference**
DNA sequence analysis	16S rDNA	Species identification, phylogenetic analysis	[61]
	gyrB	Species identification, phylogenetic analysis	[65]
	recA	Species identification, phylogenetic analysis	[66]
	rpoB	Species identification, phylogenetic analysis	[62, 63]
	16S–23S spacer rDNA	Acb complex species identification	[67]
PCR-RFLP	16S rDNA (ARDRA)	Species identification	[56, 57]
Multiplex PCR	*gyrB*	Acb complex species identification	[69]
Ribotyping	rDNA and adjacent regions	Acb complex species identification, typing	[89, 90]
AFLP analysis	Whole genome	Species and clone identification, typing	[4, 16, 60]
Multilocus sequence typing (MLST)	Multiple housekeeping genes	Assessing population diversity, clone identification	[44, 93, 94]
Multiplex PCR	*csuE,ompA, bla*$_{\text{OXA-51}}$-like gene	EU clone I–III identification	[45]
(RAPD and rep-)PCR fingerprinting	Whole genome	Typing	[41, 83, 84]
Macrorestriction analysis with PFGE	Whole genome	Typing	[87]
MLVA-VNTR	Repeats at different loci	Typing	[97]

*Typing = identification at strain level
**References of pioneer papers or of well-validated studies

Acinetobacter species [53, 54], but the method needs to be further validated with sets of reference strains of all species. If successful and if transportable between laboratories, spectra of multiple reference strains per species can be enrolled into the commercial databases.

25.9.2 Genotypic Identification Methods

DNA-DNA hybridization (DDH) assesses the overall nucleotide similarity between strains and is the gold standard in taxonomy to delineate species [55]. Three pioneer studies of the 1980s based on DDH of extensive numbers of strains have laid the foundation for the current taxonomy of the genus *Acinetobacter* [11, 13, 14]. These comprehensive studies have provided the basic reference collections for the development of alternative methods for identification. From the 1990s onward, various

genotypic methods for *Acinetobacter* species identification have been described, a selection of which will be discussed in this chapter (Table 25.2). Initial methods are based on the comparison of profiles of DNA fragments that differ in size. These fragments are in most cases generated by PCR amplification and/or digestion of DNA with restriction enzymes and are separated according to size by electrophoresis. After visualization, profiles are compared to those of reference strains of the different species for identification. Fragment-based methods including e.g. ARDRA and AFLP analysis, although still valuable for species identification, are now increasingly being replaced by sequencing-based methods. Once particular sequences are known to be specific for a species, (multiplex-)PCRs can target these sequences for species identification.

25.9.2.1 ARDRA

Amplified 16S ribosomal DNA restriction analysis (ARDRA) is based on restriction analysis of the amplified 16S rRNA gene [56, 57]. It belongs to the category of PCR-restriction fragment length polymorphism (RFLP) detection methods. Crude DNA, prepared by alkaline cell lysis, is used for PCR amplification of the 16S rDNA sequence. Separate fractions of the amplified product are digested with five restriction enzymes (CfoI, AluI, MboI, RsaI, MspI). Restriction products are separated by agarose gel elctrophoresis. For identification, the combined patterns ("profiles") of each strain are compared visually to a library of profiles of strains of all species [57]. Multiple profiles occur in some species, while identical profiles occur in several species. In the latter case, additional phenotypic testing is required for definitive identification ("consensus identification") [49]. Despite these limitations, ARDRA is easy to perform and results can be compared between laboratories. Since the original description of the method [56, 57], the library of the Leiden University Medical Center has been extended with numerous profiles including those for the two novel species, *A. gyllenbergii* and *A. beijerinckii* [58], emphasizing the heterogeneity of the 16S ribosomal gene in *Acinetobacter*.

25.9.2.2 AFLP Analysis

AFLP™ analysis is another genotypic method for *Acinetobacter* species identification. The method is essentially a restriction-based method and pioneered for bacteria by Janssen et al. [59]. The method comprises the following steps: digestion of bacterial DNA with two restriction enzymes, ligation of adaptors to the restriction fragments, selective amplification of restriction fragments, electrophoretic separation of fragments, and visualization and analysis of profiles. At the Leiden University Medical Center, AFLP analysis has been used extensively for characterization of microorganisms including *Acinetobacter*. EcoRI and MseI are the enzymes used to digest genomic *Acinetobacter* DNA in one step with adapter ligation. A Cy-5 labelled EcoRI + A and Mse + C (A, C = selective nucleotides)

primer are used for amplification, and the obtained fragments are separated on the ALFII express sequencing machine (GE Healthcare, Roosendaal, Netherlands) with automated laser detection. Images in Tiff format are used for pattern analysis using BioNumerics software (Applied Maths, Sint-Martens- Latem, Belgium) with Pearson's product moment correlation coefficient as a similarity measure and the unweighted pair group average linkage method (UPGMA) for grouping. Visual inspection of patterns and grouping is an essential part of the analysis. Grouping of isolates identified by DDH corresponded generally well with AFLP grouping at a similarity level of 50% [59, 60], which was considered the species cut-off level. A database of ca. 2,500 *Acinetobacter* isolates has been set up and the method has appeared powerful to identify isolates to species and delineate novel species. Within a laboratory and for a given sequencing platform the method is robust, but comparison of profiles between laboratories is not possible. Fragment separation with capillary sequencing systems is the way ahead for high-throughput testing of strains using AFLP.

25.9.2.3 Sequencing-Based Methods

16S rRNA gene sequence analysis. The 16S rRNA gene sequence is widely used in clinical practice for species identification including *Acinetobacter* species. A detailed description of the method and its usefulness for *Acinetobacter* identification has been provided by Vaneechoutte et al. [61]. By this method, the PCR-amplified target sequence (a DNA fragment of the 16S rRNA gene corresponding to the positions 10–1,507 of the *Escherichia coli* numbering system) is compared to those in a (public) database to assess its taxonomic (phylogenetic) position, i.e. the species it is closest to. It is important to note that public databases may be contaminated with sequences of organisms of unclear taxonomic position or with incorrect sequences. Therefore, quality-controlled sequence databases may have preference (e.g. Ridom, http://www.ridom.de). Although 16S rDNA sequence analysis is a valuable method, the intra- and interspecies similarity values are very close (≥99.7% vs. ≤99.6%). Furthermore, the grouping of some *Acinetobacter* species including those of the Acb complex obtained by this method does not correspond well with the grouping by DDH [61].

Sequence analysis of rpoB. The RNA polymerase B (rpoB) subunit gene has been studied in most detail for Acinetobacter species identification. La Scola et al. [62] tested four zones within or flanking the gene and found two polymorphic zones ("zones 1 and 2") that were particularly promising. With an extended set of reference strains it was further shown that zone 1 alone was sufficient for identification of the four main Acb species [63]. The combined zones 1 and 2 (861 bp) were also useful for identification of these species (Fig. 25.1) and to delineate several other novel *Acinetobacter* species [6, 53, 64]. One exception was the grouping of the type strain of *A. baylyi* with *A. guillouiae* (former genomic species 10) [53]. This might be due to intragenic recombination following acquisition by *A. baylyi* of rpoB sequences from *A. guillouiae*, since *A. baylyi* is known to be transformable by *Acinetobacter*

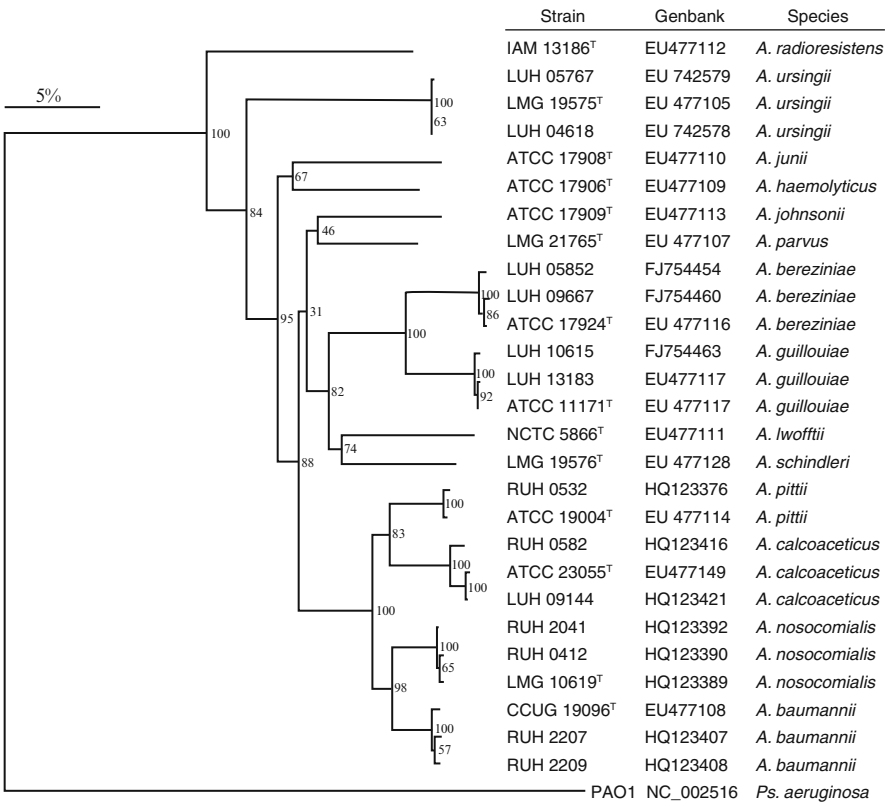
Strain Genbank Species
5%
IAM 13186T EU477112 A. radioresistens
LUH 05767 EU 742579 A. ursingii
LMG 19575T EU 477105 A. ursingii
LUH 04618 EU 742578 A. ursingii
ATCC 17908T EU477110 A. junii
ATCC 17906T EU477109 A. haemolyticus
ATCC 17909T EU477113 A. johnsonii
LMG 21765T EU 477107 A. parvus
LUH 05852 FJ754454 A. bereziniae
LUH 09667 FJ754460 A. bereziniae
ATCC 17924T EU 477116 A. bereziniae
LUH 10615 FJ754463 A. guillouiae
LUH 13183 EU477117 A. guillouiae
ATCC 11171T EU 477117 A. guillouiae
NCTC 5866T EU477111 A. lwofftii
LMG 19576T EU 477128 A. schindleri
RUH 0532 HQ123376 A. pittii
ATCC 19004T EU 477114 A. pittii
RUH 0582 HQ123416 A. calcoaceticus
ATCC 23055T EU477149 A. calcoaceticus
LUH 09144 HQ123421 A. calcoaceticus
RUH 2041 HQ123392 A. nosocomialis
RUH 0412 HQ123390 A. nosocomialis
LMG 10619T HQ123389 A. nosocomialis
CCUG 19096T EU477108 A. baumannii
RUH 2207 HQ123407 A. baumannii
RUH 2209 HQ123408 A. baumannii
PAO1 NC_002516 Ps. aeruginosa

Fig. 25.1 Rooted neighbor-joining tree based on partial nucleotide sequence of *rpoB* (position 2,915–3,775) of sets of reference *Acinetobacter* strains of the same species and single strains of several other species showing high similarity intraspecies similarity. All strains were previously identified by validated methods including DNA-DNA hybridization, AFLP analysis, and/or ARDRA. Bootstrap percentages after 1,000 resampling are given at nodes. Bar, 5% sequence diversity

DNA. Although not yet validated for all described species, the rpoB sequence is currently the most promising sequence for *Acinetobacter* species identification.

Sequence analysis of other genes. Apart from the 16S rRNA and *rpoB* gene sequence, a variety of other sequences have been investigated for their potential as taxonomic marker. Of these, *gyrB* [65] was in good agreement with DDH classification for 18 (geno)species. The *recA* gene was used for phylogenetic analysis of the genus, and a classification based on restriction profiles of this sequence generated with Tsp5091 enzyme was proposed as a species identification scheme [66]. The 16–23 S rRNA intergenic spacer region [67] appeared useful for identification and differentiation of the species in the Acb complex [67]. Seven house-keeping genes included in a system for multilocus sequence typing (MLST) [44] also grouped isolates according to species, emphasizing that a variety of genes are potentially useful for *Acinetobacter* species identification.

(Multiplex-)PCR methods detecting species-specific gene sequences. In a study of 144 *A. baumannii* strains and ten non-*baumannii* strains, $bla_{\text{OXA-51}}$-like, the intrinsic carbapenemase gene, was found to be specific for *A. baumannii* as detected in a multiplex-PCR targeting also $bla_{\text{OXA-23-like}}$ and the class 1 integrase gene [34]. However, $bla_{\text{OXA-51}}$-like has since been detected in Asian strains of *A. nosocomialis*, indicating that it may not always be specific for *A. baumannii* [17, 68]. Multiplex-PCR identification based on specific sequences in the *gyrB* gene has been developed for identification of the four species of the Acb complex [69].

25.10 Methods for Typing Acinetobacters

Typing is defined here as the approach to differentiate organisms below species level. It can be done to assess strain relatedness at the local (hospital) level in case of suspected cross-infections or to assess the sources and mode of transmission of an outbreak strain. It can also be done at the regional or international level to investigate the geographical diversity of the species and the spread of particular strains or clones. Depending on the purpose, different methods are required. For example, for quick assessment of genotypic relatedness of a few isolates in a hospital, PCR fingerprinting can be sufficient. However, if large numbers of strains are to be compared either locally over prolonged periods or between institutes, more robust methods are required, the results of which can be stored in digital databases for longitudinal or interlaboratory comparisons. In the following section, an overview of typing methods is given. Guidelines for strain typing have been issued by the European Society for Clinical Microbiology and Infectious Diseases (ESCMID) [70].

25.10.1 Phenotypic Typing Methods

Antibiotic susceptibility profiles are the first character in diagnostic microbiology to recognize strains with important resistance traits. Precise zone sizes in case of disk diffusion susceptibility testing can help to differentiate strains [38]. However, further strain characterization is usually required since unrelated strains may share particular resistances (even if the responsible genes are different) and related strains may lose or gain genes conferring resistance.

Further to antibiogram typing, a variety of methods have been used for phenotypic typing of acinetobacters. Phage typing, a high-throughput method, has been successfully used to type *A. baumannii* strains [71] but was applied mainly at one reference laboratory. A biotyping system based on differential growth on six carbon substrates has been described [72], but it does not allow for differentiation at the strain level [43]. Cell-envelope SDS-PAGE protein profiling has been used for strain and clone identification in the 1980s and 1990s [23, 38] but only at one institute.

More recently, provisional results with Raman spectroscopy have shown that fingerprints, which are a reflection of the chemical make-up of the whole cell (including outer membrane proteins), can be used for rapid typing of *Acinetobacter* strains [73]. Serotyping using monoclonal antibodies targeting the O-antigen of the lipopolysaccharide of acinetobacters has indicated a great diversity in this surface-located antigen [74], but no attempts have been made yet to translate this approach to a practical assay.

25.10.2 Genotypic Typing Methods

25.10.2.1 Plasmid Typing

From the late 1980s up to the mid 1990s, plasmid typing was frequently applied in hospital epidemiology, often in combination with other methods. Within the Acb complex a high intraspecific diversity of plasmids was found [75–77]. Genes on plasmids may confer resistance to antibiotics or metabolic properties, but for most plasmids their function is yet unknown. Most indigenous plasmids of *Acinetobacter* spp. are relatively small (<23 kb), which makes it unlikely that they are conjugative [78]. A genomics approach showed that although plasmids lack mobilization functions, they have probably a long history of exchange with other plasmids and chromosomes [79]. Recent studies have indicated the spread of plasmid-associated carbapenem resistance genes [80, 81]. A special application of plasmid typing is PCR-based replicon sequence typing to characterize *A. baumannii* strains with a $bla_{\text{OXA-58}}$ or $bla_{\text{OXA-23}}$ carbapenemase gene [82]. With this approach, a conjugative plasmid was identified that was responsible for the spread of the resistance genes.

25.10.2.2 PCR Fingerprinting

Simple, comparative typing of strains can be done by PCR fingerprinting. Examples are random PCR amplification of polymorphic DNA fragments (RAPD) or repetitive-sequence-based PCR (rep-PCR) amplification of regions between the noncoding repetitive sequences in bacterial genomes. Primer sites can vary in number and location over the genome and, consequently, amplified fragments vary in number and sizes. A multicenter group has evaluated the intra- and interlaboratory reproducibility of PCR-based fingerprinting of *Acinetobacter* strains using quality controlled reagents and a standardized protocol [83]. The pattern of grouping strains was comparable between laboratories, but interlaboratory similarity of profiles was low ($S \geq 0.7$). Attempts to improve the reproducibility were not successful (Seifert, Dolzani and Dijkshoorn, unpublished results). Nevertheless, the protocol with primers DAF4 and M13 appeared useful for local study of strain relatedness [84]. Profiles can be compared visually if samples are in adjoining lanes or by computer assisted analysis using Pearson's correlation coefficient and UPGMA. PCR

fingerprinting with the repetitive (GTG)5-primer also allowed for differentiation of *A. baumannii* strains and identification of the three major European clones I–III [85]. Altogether, PCR fingerprinting is a rapid and easy method for local typing but is, as a manual method, not useful for longitudinal or interlaboratory comparison. The automated rep-PCR system, Diversilab™ (bioMérieux, Marcy l'Etoile, France) [86], which separates fragments by microfluidics electrophoresis followed by laser detection, is more robust. Fingerprints generated with this system were found highly discriminatory and comparable to AFLP analysis to type large sets of *A. baumannii* strains [41, 86].

25.10.2.3 Macrorestriction Analysis with Pulsed Field gel Electrophoresis (PFGE)

Whole genomic fingerprinting using PFGE is based on digestion of genomic DNA with a rare-cutter restriction enzyme that generates relatively large fragments. The fragments are separated by pulsed field gel electrophoresis with fragments migrating zig-zag wise through an agarose matrix according to an electric field that alternates periodically at an angle of (usually) 120°. Profiles can be compared visually or by computer assisted analysis using a band-based similarity coefficient such as the Dice coefficient and UPGMA or the single linkage method for clustering. PFGE has been widely applied since the 1990s in reference and diagnostic laboratories and is still an important method for epidemiological typing. A three-center study of strains of the Acb complex with a strict protocol and hands-on training of technicians has shown that PFGE with ApaI as cutting enzyme provides profiles that are comparable between laboratories [87]. The method has a high resolution, which makes PFGE useful for identification at the strain level (Fig.25.2). Results largely agree with results obtained by AFLP analysis [27, 39]. Criteria for the number of band differences to assess epidemiological relatedness of outbreak strains [88] have not been validated for acinetobacters.

25.10.2.4 Ribotyping

Ribotyping, although primarily developed for species identification [89], is a useful method for typing, mainly of Acb complex strains. Ribotyping is a Southern blotting technique, by which bacterial genomic DNA is digested with a restriction enzyme. Next, restriction fragments are separated electrophoretically on agarose gel and transferred by vacuo blotting to a filter and hybridized with a labelled probe. After visualization, the profiles are analysed either by computer assisted software or visually and compared to reference strains for species identification. An automatic ribotyping system (Riboprinter, DuPont Qualicon, Wilmington, DE) has been used in several studies for typing acinetobacters [39, 90]. Various enzymes have been used for typing acinetobacters including EcoRI, ClaI, SalI, HindIII, and HincII

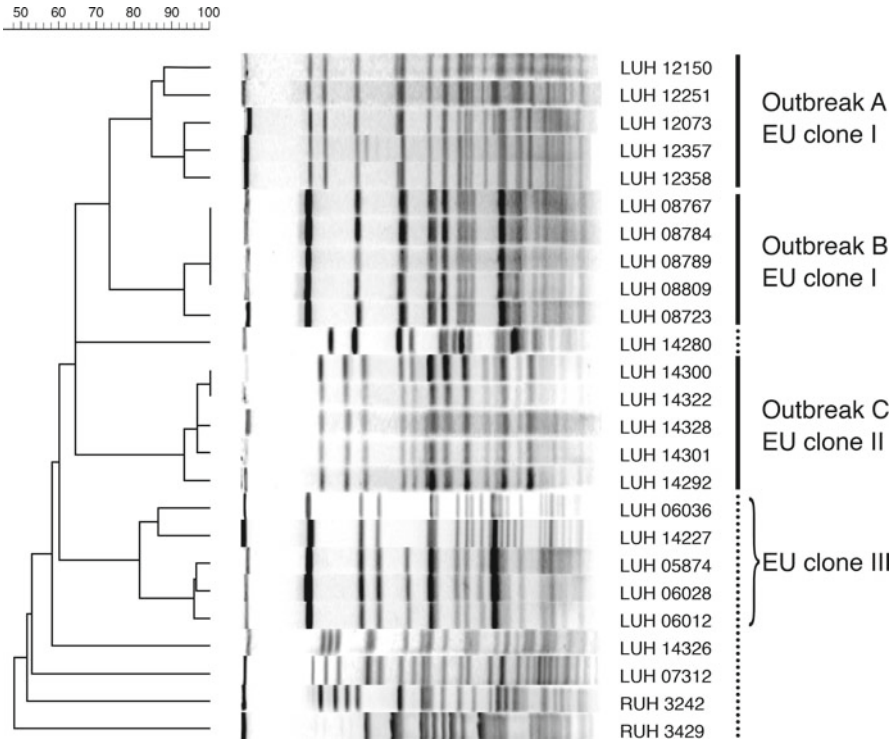

Fig. 25.2 Pulsed field gel electrophoresis profiles of *A. baumannii* isolates from three distinct outbreaks A, B, and C and identified to EU clones I and II (marked by black lines), and isolates unrelated in time and space (dotted lines). Isolates of EU clone III were highly similar in profile, indicating that results for these organisms must be interpreted with caution and preferably in association with other typing methods. ApaI was used as restriction enzyme and a standardized protocol was followed [87]

[43, 89]. Although overall grouping by ribotyping correlates well with AFLP analysis and protein typing [38], it is less discriminatory than AFLP and PFGE [39, 43].

25.10.2.5 AFLP Analysis

Characterization of acinetobacters by AFLP has, apart from being used for species identification, been applied in numerous studies to investigate the epidemiology and diversity of acinetobacters [16, 24, 37, 60, 91]. Most of these studies have been performed at one institute, the LUMC. Isolates can be either compared to each other without no reference to other strains to assess relatedness, e.g. for outbreak analysis, or compared to isolates in the LUMC database containing strains from different time-space origin. Studies with sets of epidemiologically well-defined strains have revealed that multiple isolates from an outbreak or isolates from single patients are similar well above 90% [16, 91], a level used as the

strain cut-off level. Furthermore, AFLP has been instrumental in delineating clusters of isolates within *A. baumannii* at a similarity level of ≥80%, which are considered to represent clonal lineages [38, 60, 92]. It is important to note that this cut-off level is relative. With the growing number of isolates of these clones representing a wide time-space origin, isolates are found that link with the clones between 70 and 80% (Fig. 25.3). Although AFLP requires experience and strict standardization, it is a robust method with a broad identification range, i.e., from strain to species level. If combined with capillary electrophoresis, the method has the potential of a high-throughput method for reference institutes, but transportability between institutes will be difficult due to differences in sequencing platforms.

25.10.2.6 Multilocus Sequence Typing (MLST)

Comparative analysis of organisms according to similarity in sequences of internal fragments of protein encoding housekeeping, MLST, has become the standard for investigation of the population diversity of bacterial species. For each gene fragment, the different sequences are assigned as distinct alleles and each isolate is defined by the combination of alleles for each locus. Three systems have been described for *Acinetobacter*. One system [93] comprises multilocus PCR followed by electrospray ionization mass spectrometry (PCR/ESI-MS), which uses the amplicon base compositions (numbers of A, T, C, G) of amplified gene sequences for typing. Analysis time is 4 h only. Six housekeeping genes were used to type 267 isolates of *Acinetobacter.* Results were largely in agreement with PFGE typing and the approach also appeared useful for species identification.

Another MLST system (http://pubmlst.org/abaumannii/) comprises 305–513 bp internal fragments of seven housekeeping genes. In two studies comprising strains from Spain and Germany [94] and from outbreaks in Europe and the USA [95], 65 sequence types (STs) were distinguished among 96 *A baumannii* strains. E-burst analysis identified five clonal complexes (CCs) and 55 singletons among the organisms. The system was also useful for *A. nosocomialis* as 20 isolates of this species were identified by this system to 14 singletons and two CCs.

A third system (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html) [44] also used internal fragments of seven housekeeping genes including three genes of the second system. For validation, a diverse collection of *A. baumannii* strains including sets from outbreaks, isolates representing the three EU clones, sporadic isolates, and sets of other *Acinetobacter* species was used. Results indicated a good correlation of MLST with previous AFLP typing of the strains, in particular regarding identification of the EU clones I–III, that were by MLST allocated to clonal complexes 1–3 (Fig. 25.3). However, it seems that the system is less discriminatory than AFLP analysis and the second MLST system. Integration of the two systems should be considered to optimise its application range.

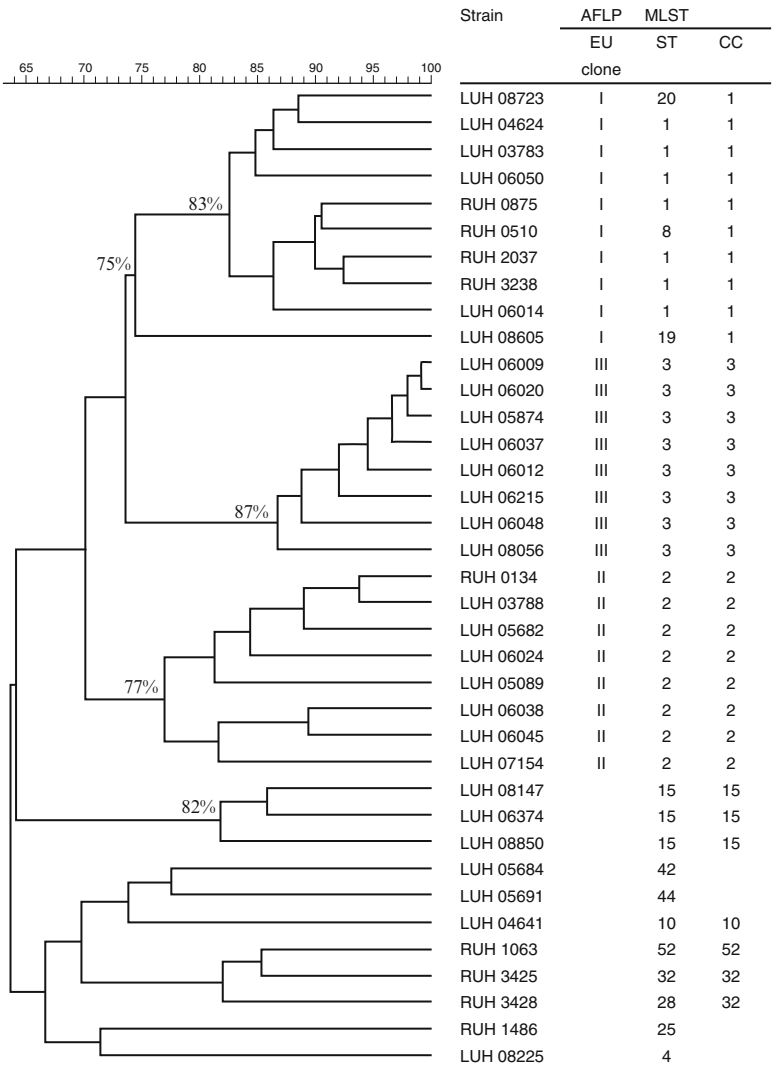

Fig. 25.3 AFLP analysis of *A. baumannii* isolates of EU clone I–III and other isolates and comparison of results of MLST [44]. Arrows denote the AFLP cut-off levels of 80 and 90% generally used for clone and strain identification (see also text). Grouping correlated well with allocation of isolates by MLST to clonal complexes (CCs). Technical details of the AFLP method are given elsewhere [16]

25.10.2.7 Variable Number of Tandem Repeat (VNTR) Analysis

A novel development in bacterial typing is based on the determination of the variable number of tandem repeats of particular DNA motifs in a genome. Multiple-locus VNTR analysis (MLVA) is becoming an important method for bacterial strain

typing. First, a bioinformatic approach is used to detect putatitve VNTRs in genome sequences and to design primers flanking the regions to develop PCRs for amplification of the repeats. The sizes of the fragments, corresponding to the number of repeats, are used for differentiation organisms. Turton et al. [96] pioneered this approach for *A. baumannii* and identified two loci that were useful in combination with PFGE to trace transmissions between patients. Recently, Pourcel et al. [97] presented a scheme based on allele differences at eight loci. For each locus, amplicons were separated by agarose electrophoresis, and each strain was assigned a code representing the number of repeats per locus. Results allowed for EU clone identification, and the MLVA grouping corresponded well with grouping by PFGE and PCR-based profiling of *ompA*, *csuE*, and $bla_{\text{OXA-51}}$-like genes to identify EU clones, and it was more discriminatory than the latter two methods. MLVA data can be stored in a database and exchanged between institutes (http://mlva.u-psud.fr). A further step in this development will be the design of multiplex PCRs and fragment separation by capillary electrophoresis as developed for other microorganisms [98].

25.10.2.8 Other Genotypic Typing Methods

Several other methods have been used for *Acinetobacter* strain characterization. For example, rapid identification of *Acinetobacter baumannii* to the European clones I–III can be obtained by a multiplex PCR targeting the genes *ompA*, *csuE*, and $bla_{\text{OXA-51}}$-like [45]. Size differences among alleles of each gene sequence could be detected by electrophoretic separation.

The emergence of carbapenem resistance in *A. baumannii* frequently requires detailed characterization of the organism to answer regional or local questions on the genetic basis of the carbapenem resistance. It is therefore becoming current practice to supplement typing by PCR-detection of genes associated with carbapenem resistance [35, 99]. Due to the highly clonal nature of *A. baumannii*, the methods described in the previous paragraphs may be insufficient for cross-infection studies. Novel approaches, targeting useful markers found by exploring sequenced genomes, have opened new windows in this area [100, 101].

25.11 Conclusions and Recommendations

MDR *A. baumannii,* and, to a lesser extent, *A pittii* and *A nosocomialis* are an increasing problem in the clinical setting, and their global emergence is worrying. Methods for identification at the species, strain, and clonal level are important to study their behaviour and the sources and modes of transmission. A wide repertoire of methods is now available to meet this need.

With the increasing availability of DNA sequencing facilities, *rpoB* sequence analysis is probably the easiest way for confirmative species identification. It is

likely that MALDI-TOF MS will soon be a widely available alternative for rapid species identification.

PCR fingerprinting and PFGE are useful and relatively easy methods for local strain typing, for example to detect cross-infections. For reference laboratories or for local, longitudinal strain monitoring, robust methods including standardized PFGE, AFLP, or Diversilab Rep-typing can be used to generate local reference databases. MLST is important to assess the population diversity including identification of isolations to the EU clones I–III. These clones can also easily be identified by a multiplex PCR targeting three gene sequences [45]. MLVA is a promising method for its expected robustness and transportability between laboratories, which will enable microbiologists to identify their organisms with those of an Internet-based library. For the study of complex epidemiological situations, it is recommended not to rely on a single method but to use combinations of methods for strain identification and to interpret results within the context of the epidemiological background of the organisms.

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Chapter 26
Molecular Typing of *Coxiella burnetii*: A Review of Available Methods with Major Focus on PCR-Based Techniques

V. Boldis, E. Spitalska, and R. Toman

26.1 Introduction

Coxiella burnetii is an obligate intracellular, small Gram-negative bacterium. Although possessing a membrane similar to that of a Gram-negative bacterium, it is usually not stainable by the Gram technique. The Gimenez method is predominantly used to stain the bacterium in clinical specimens or laboratory cultures. In the past, *C. burnetii* was classified in the *Rickettsiales* order but more recent phylogenetic investigations, based mainly on 16S rRNA sequence analysis, resulted in a re-classification of the *Coxiella* genus to the γ-subdivision of *Proteobacteria* within the *Legionellales* order and family *Coxiellaceae*. Since 2003, five whole genome sequencing projects of different *C. burnetii* isolates have been completed with two additional isolates currently in assembly. The circular genome of the Nine Mile RSA 493 isolate has a length of 1,995,275 base pairs (bp, [1]).

C. burnetii is highly infectious and causes Q fever, a zoonotic disease, which is capable of being transmitted from animals to humans [2, 3]. In humans, the most common acute form of Q fever is manifested as a self-limited febrile illness or pneumonia, or less frequently as hepatitis [2]. Persistent infection in humans can lead to a chronic form of Q fever, which may be associated with a fatal endocarditis [2]. In animals, Q fever affects livestock and is associated with pneumonia and reproductive disorders in livestock, with abortion, stillbirth, placentitis, endometritis, and infertility [3]. The main route of infection is inhalation of contaminated aerosol or dust containing bacteria shed by infected animals through milk, feces, placental, or

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vaginal secretions. An easy aerosol dissemination, environmental persistence, and high infectivity make the bacterium a serious threat for military personnel and civilians [4]. This was also the justification for its inclusion in the CDC list of potential bioterrorism agents. Oral transmission seems less common, but the consumption of contaminated raw milk and dairy products represents a potential source of human infection [3]. As several clinical symptoms of Q fever are similar to commonly occurring infections, an unambiguous diagnosis of the disease is quite difficult.

Currently, serological methods are most frequently used in diagnosing Q fever. In part, this is often a result of time delays before clinicians or veterinarians consider the possibility of Q fever occurrence and also of the fact of providing a cost-effective method compared with molecular diagnostics. Using specific anti IgM-, IgG-, and IgA- antibodies, both ELISA or indirect immunofluorescence tests are usually used to characterize acute or persistent/chronic infections with *C. burnetii* [5, 6]. It must be kept in mind, however, that all serological tests could potentially cross-react with antigenically related species such as *Legionella*, *Bartonella*, and *Chlamydophila/Chlamydia.* With the introduction of molecular techniques, remarkable improvements in the diagnostic capabilities of *C. burnetii* (Q fever) have been achieved. In fact, many laboratories complement serological data with the increasing number of molecular biology techniques, especially by PCR. Thus, availability of the whole genome sequence data of *C. burnetii* has enabled the design and application of sophisticated, high-resolution molecular typing systems that are described in this chapter.

26.2 Characterization of Plasmid Type

At the molecular level, different isolates of *C. burnetii* can be characterized by their plasmid types. *C. burnetii* isolates appear with five different plasmids, independent from the phase-associated lipopolysaccharide modifications. Five plasmids (QpH1, QpRS, QpDG, QpDV and a plasmid without designation derived from a Chinese *C. burnetii* isolate) and one plasmidless type have been found in the bacterium [7, 8]. Characterization of these plasmids led to the classification of *C. burnetti* into six genomic groups. The QpH1 plasmid was first obtained from a tick isolate and was also detected in most isolates originating from ticks, domestic animals (cows, goats, and sheep), and acute Q fever patients. The QpRS plasmid was first detected in an isolate from an aborted goat and was then found in most isolates from patients with chronic Q fever. The QpDG plasmid was found in only a few isolates from wild rodents. The QpDV plasmid was discovered in an isolate from cow's milk and an isolate from a human with pneumonia and was also found in three isolates from patients with acute Q fever, an aortic aneurysm, and chronic endocarditis, respectively. In several isolates from humans with endocarditis, a separate plasmid DNA was not isolated, but the plasmid sequences were integrated into the chromosomes of these isolates. Identification of *C. burnetii* plasmids may provide some basic information in the differential diagnosis of Q fever and in epidemiological investigations. However, a correlation of six genomic groups of *C. burnetii* with its virulence or clinical manifestations could not be proved [9].

26.3 Restriction Fragment Length Polymorphism Analysis

Variations in the DNA sequence of a genome can be detected by the restriction fragment length polymorphism (RFLP) analysis based on restriction of the DNA into fragments with restriction enzymes (RE) and analyzing the size of the resulting fragments by gel electrophoresis (GE).

Heinzen et al. [10] examined isolates of *C. burnetii* using pulsed-field GE, and the RE Not I and Sfi I gave the fewest and most easily resolved fragments. In this way, four different genomic groups (I, IV, V, and VI) could be established. Thirty-two isolates of *C. burnetii*, collected from various hosts ranging from arthropods to man, were compared [11] by the RE EcoRI and BamHI digestion patterns of chromosomal DNA using polyacrylamide GE in sodium dodecyl sulphate (SDS-PAGE). This provided better DNA fragment separation compared to agarose GE and enabled differentiation of the isolates into six distinct genomic groups (I–VI) on the basis of DNA restriction fingerprints. Later, Thiele et al. [12] could find five additional DNA banding patterns from European and one Namibian isolates of *C. burnetii* after endonuclease restriction with Not I. Finally, 20 different restriction patterns could be distinguished in the isolates originating from animals and humans in Europe, USA, Africa, and Asia [13] after Not I digestion. Thus, the RFLP analysis led to a classification of *C. burnetii* isolates into six groups (I–VI) very similar to plasmid typing. This method, however, has not brought unambiguous results in the etiology and pathogenicity of the infectious agent.

26.4 Polymerase Chain Reaction Based Diagnostic Assays

Polymerase chain reaction (PCR) based diagnostic assays have been developed to detect *C. burnetii* DNA mainly in the cell cultures and clinical samples. These assays use conventional PCR, nested PCR, or real-time PCR conditions with a LightCycler, SYBR Green, or TaqMan chemistry.

26.4.1 Conventional PCR

PCR is a technique to amplify copies of a fragment of DNA across several orders of magnitude, generating millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Key components enabling selective and repeated amplification are the sequences complementary to the target region (primers, short DNA fragments) and a DNA polymerase. During PCR, the DNA generated is itself used as a template for replication. Sets of commonly used primers were designed using, e.g. superoxide dismutase (SOD), *com1,* 23S rDNA, and *IS1111* genes for the detection of *C. burnetii* based on conventional PCR.

A pair of primers (CB1 [5′-ACT CAA CGC ACT GGA ACC GC-3′] and CB2 [5′-TAG CTG AAG CCA ATT CGC C-3′]) derived from the *C. burnetii* SOD gene amplify a targeted 257-bp fragment of genomic DNA. This technique allows detection of as few as ten *C. burnetii* organisms. The bacterium is detected in tissue culture and in specimens from patients (heart valves). The amplification products of *C. burnetii* are confirmed by the RE digestion with AluI and TaqI. The AluI sites produced three fragments of 186, 68, and 3 bp and the TaqI sites generated four fragments of 118, 57, 43, and 39 bp [14].

On the basis of the nucleotide sequence data, the primers CBCOS [5′-GCT GTT TCT GCC GAA CGT AT-3′] and CBCOE [5′-AGA CAA CGC GGA GGT TTT TA-3′] were synthesized for a highly conserved gene *com1* among *C. burnetii* isolates, which encodes a 27-kDa outer membrane-associated immunoreactive protein. Primers were designed to amplify the nucleotide positions at 186–679 bp. The expected product contained single MspI and Sau3AI restriction sites and was predicted to be digested to 359 and 135 bp, and 395 and 99 bp fragments, respectively. This method was used, for example, in the detection of *C. burnetii* in ticks [15].

A primer set 976F [5′-AGG TCC GTG GTG GAA AGG AAC G-3′] and 1446R [5′-TCT CAT CTG CCG AAC CCA TTG C-3′] was designed to amplify a 477-bp fragment encompassing a part of the intervening sequence and a part of the 23S rDNA. The RE digestion of a PCR product from the *C. burnetii* isolates with Rsa I gave two distinct fragments approximately 210- and 270-bp in size. The method showed a detection limit of 10^2 bacteria and proved to be specific for the bacterium [16].

The *IS1111*-insertion sequence, coding for a transposase, is present in as many as 52 copies in *C. burnetii* genomes. Therefore, this element is often used as a specific target region in highly sensitive diagnostic PCR. The method was used for the detection of *C. burnetii* in cow's milk and clinical samples. The trans-1 [5′-TAT GTA TCC ACC GTA GCC AGT C-3′] and trans-2 [5′-CCC AAC AAC ACC TCC TTA TTC-3′] primers were designed to amplify a 687-bp fragment. By applying this method, 10^0 templates could be detected with a high reproducibility [17]. Furthermore, the trans-3 [5′-GTA ACG ATG CGC AGG CGA T-3′] and trans-4 [5′-CCA CCG CTT CGC TCG CTA-3′] primers were designed to amplify a 243-bp fragment of a transposon-like repetitive element to detect the bacterium in clinical samples, such as placental bits, genital and fecal swabs, urine, liver, spleen, placenta, heart valves, milk, blood, and serum samples [18, 19]. The analytical sensitivity of the Trans-PCR was found to be 10^0 (sometimes even 10^{-1}) *C. burnetii* particles per reaction mixture.

26.4.2 Nested PCR

The nested PCR approach was applied for highly sensitive and specific direct detection of *C. burnetii*. Primers OMP1 [5′-AGT AGA AGC ATC CCA AGC ATT G-3′] and OMP2 [5′-TGC CTG CTA GCT GTA ACG ATT G-3′], OMP3 [5′-GAA GCG

CAA CAA GAA GAA CAC-3′], and OMP4 [5′-TTG GAA GTT ATC ACG CAG TTG-3′] were designed from the nucleotide sequence of the *C. burnetii com1* gene. Amplifiers used for *C. burnetii* amplification of DNA extracted from the blood samples from patients with chronic nonspecific symptoms yielded 501- and 438-bp fragments [20]. Further, the method was used for a direct identification of *C. burnetii* plasmids in human sera. The first set of primers OMP1, OMP2, OMP3, and OMP4 was used to detect the genomic sequences. The second set HFrag1 [5′-ATT GCT ATC ACT GAG GGT GAC G-3′], HFrag2 [5′-CTG ACG AAG AAG CAG CAT TAG C-3′], HF1 [5′-TCC TAA ACA AGT GAT GGT CTC C-3′], and HF2 [5′-TTC GCA GAA AGT CAG CTA TCG-3′] was applied for detecting the conserved plasmid sequences. This region is present in all types of the *C. burnetii* plasmids. Two sets of modified primers were used to detect the *C. burnetii* plasmid-specific sequences. The first one, CB5–CB6 [5′-ATA ATG AGA TTA GAA CAA CCA AGA-3′, 5′-TCT TTC TTG TTC ATT TTC TGA GTC-3′] and CB3–CB4 [5′-TAA TAG AAC GTG TTA ATC G-3′, 5′-GCT GGC AAT CTG CTC GGC-3′] was designed from a specific gene of the QpH1 plasmid, *cbhE*9. The second, QpRS1–QpRS2 [5′-CTC GTA CCC AAA GAC TAT GAA TAT ATC C-3′, 5′-AAC ACC GAT CAA TGC GAC TAG CCC-3′] and QpRS3–QpRS4 [5′-ACT TTA CGT CGT TTA ATT CGC-3′, 5′-CAC ATT GGG TAT CGT ACT GTC CCT-3′] was created from a unique gene of the QpRS plasmid, *cbbE*9. Primers HFrag1–HFrag2 and HF1–HF2 yielded 508 and 183 bp fragments and the primers CB5–CB6 and CB3–CB4 amplified the expected products of 977 and 266 bp, respectively. The primers QpRS1–QpRS2 and QpRS3–QpRS4 gave the predicted products of 693 and 309 bp, respectively [21].

26.4.3 Real-Time PCR

Real-time (RT)-PCR, also called quantitative RT-PCR, is used to amplify and simultaneously to quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of a specific sequence in a DNA sample. The target sequences of the assays in the *C. burnetii* field originate from the singular chromosomal genes such as *com1*, *htpB*, and *icd*, a gene encoding isocitrate dehydrogenase, and of those sequences present on plasmids (QpH1 and QpRS) or on the transposase gene (*IS1111*). As mentioned earlier, *IS1111* is found in multiple copies, and several commercial test kits utilize this element to detect the bacterium by the RT-PCR techniques in just a few hours [22, 23]. Moreover, application of a Light-Cycler as the thermal cycler in RT- PCR offers in addition to rapidity several other advantages over the traditional detection techniques including elimination of subjectivity, reduced labor, and the ability to standardize reporting of results.

Further development in RT-PCR represented an application of SYBR Green I dye in the assay as shown in Fig. 26.1. It has been used for the antibiotic susceptibility testing of *C. burnetii* and provides a rapid and sensitive method for the determination

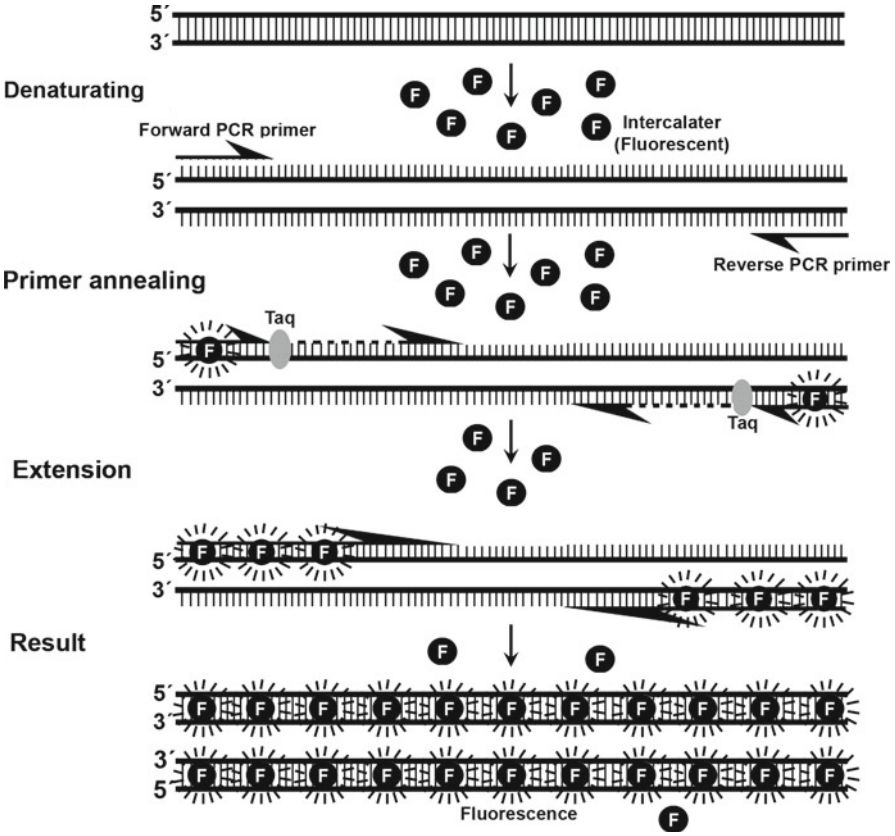

Fig. 26.1 Scheme of SYBR Green I dye-based RT-PCR assay

of *C. burnetii* antibiotic sensitivities and eliminates the subjectivity associated with the microscopic and serologic enumeration methodologies. For this purpose, the primers FAF216 [5′-GCA CTA TTT TTA GCC GGA ACC TT-3′] and RAF290 [5′-TTG AGG AGA AAA ACT GGA TTG AGA-3′], which amplify a 74-bp fragment of the *C. burnetii com1* gene, were designed [24]. To construct a standard curve (Fig. 26.2), the total genomic DNA was purified from the *C. burnetii* RSA 493 (Nine Mile) strain, and the DNA concentration was measured spectrophotometrically and converted to genome copy numbers by using the molecular weight of DNA. The tenfold serial dilutions ranging from 10^7 to 10^1 genome copies were made.

LUX (Light Upon eXtension) technology represents a new method for primer design and labeling. The first primer is marked by one fluorophore 6-carboxyfluorescein (FAM). The second is not marked. Due to the specific conformation of the marked primer as "hairpin," an interior fading of the fluorophore occurs. The connection of primer to the specific genome section prolongs the chain length and results in the enhancement of fluorophore. Kunchev et al. [25] used the primer pair CBL1

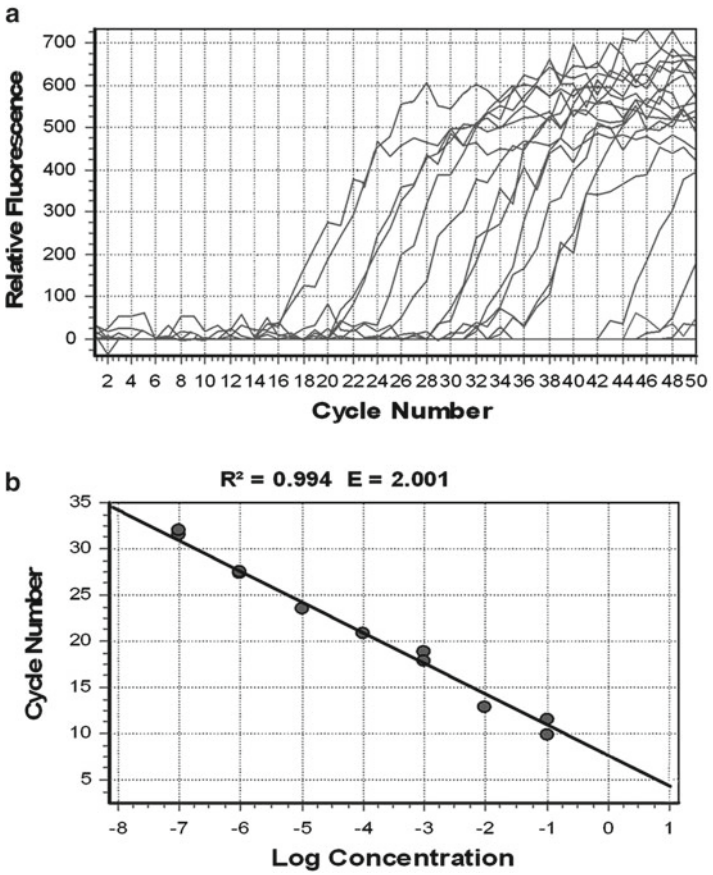

Fig. 26.2 Quantitative PCR calibration curve of the *C. burnetii com1* gene; (**a**) quantification, (**b**) standard curves

[5′-CGC ACG CTT CCA GTT CCA GTT TGT TTT G(FAM)G-3′] and CBL2 [5′-ATC AGA GCG GAC CGT CAA GC-3′] derived from the SOD gene. A high LUX primer sensitivity was evident from the fact that it detected DNA extracted from the purified *C. burnetii* corpuscular antigen in a concentration 0.587 ng/μl antigen, diluted 1:100. It appears that the LUX RT- PCR has sufficient specificity, sensitivity, and effectiveness in detecting the sequence of the *C. burnetii* genome.

Furthermore, the specificity of RT-PCR assays can be increased by the TaqMan probes that are in fact the hydrolysis probes as shown in Fig. 26.3.

Harris et al. [26] investigated patients with a chronic sequel to acute Q fever, the post-Q fever fatigue syndrome, using a sensitive, conventional target of *C. burnetii* and the TaqMan-based PCR (target: *C. burnetii IS1111a:* 61 bp; P1671f [5′-TAA CGG CGC TCT CGG TTT-3′], P2731r [5′-TGC CGG GAA CGA TGA AA-3′], probe 713-690: [5′-FAM-TGA TGA ATG TCA CCC ACG CTC GCA-TAMRA-3′

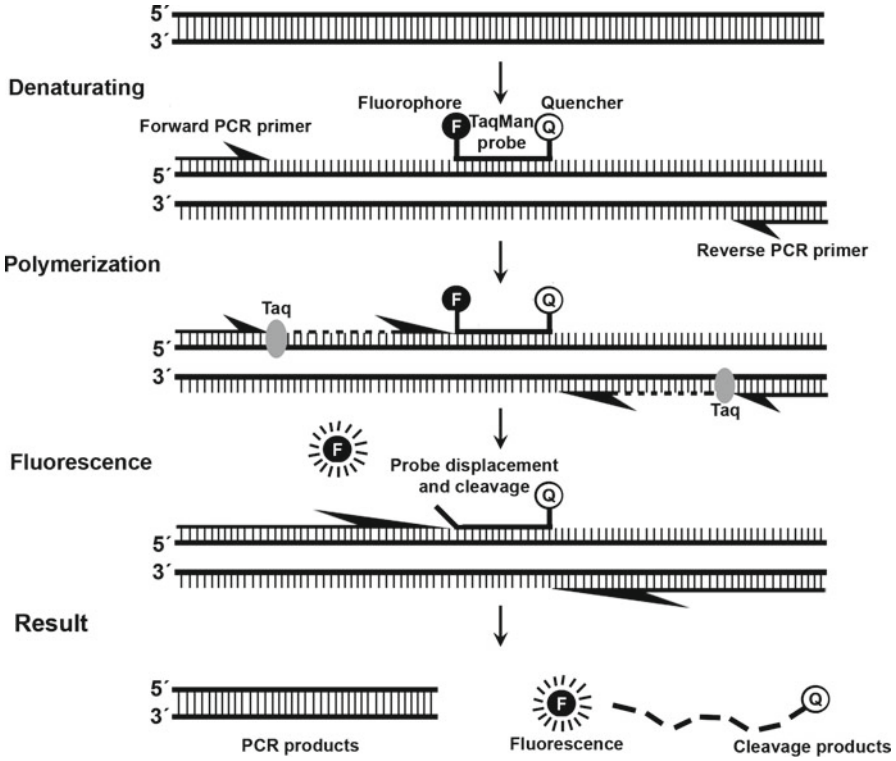

Fig. 26.3 Scheme of RT- PCR with the TaqMan probe

(PE)]). The assay revealed low levels of the *C. burnetii* DNA in blood mononuclear cells, thin needle liver biopsies, and in bone marrow aspirates. However, other studies have shown that the PCR targeting single copy genes such as *com1* and 16S rRNA genes is also sensitive enough and their use in the quantitative PCR test could be appropriate [27, 28].

Other applications of the quantitative RT-PCR assay in combination with the Balb/c mouse model may serve as a reliable and sensitive approach to evaluate the efficiency of vaccines against Q fever. The Balb/c mice were infected with *C. burnetii* and immunized with the whole cell antigen (WCA) of *C. burnetii*. Using quantitative RT-PCR specific for the bacterium by applying a 23S rRNA intervening sequence of *C. burnetii*, primer pairs CbF [5′-CGG CTG AAT TTA AGC GAT TTA TTT TT-3′] and CbR [5′-CGT AAC CAC ACA CGC ATC TCA-3′], and a TaqMan-MGB probe [5′-TGC AAT GGG TTC GG-3′]), high loads of the pathogen were found in livers, lungs, and particularly in spleens of mice. The results demonstrated that WCA was an excellent vaccinogen that elicited a complete protection against *C. burnetii* infection by two booster immunizations [29].

The TaqMan probe-based assays were also used in the research of gene expression and analysis of the morphological differentiation of *C. burnetii* [30].

26.5 Multiple Locus Sequence Typing

Different isolates of *C. burnetii* can be characterized at the molecular level also by a multiple locus sequence typing (MLST) assay. The assay is based on the intergenic region sequencing. These regions are potentially variable since they are subject to lower selection pressure than the adjacent genes. Glazunova et al. [31] screened 68 spacers in 14 isolates of *C. burnetii* and selected ten short intergenic regions, Cox2, Cox5, Cox18, Cox20, Cox22, Cox37, Cox51, Cox56, Cox57, and Cox61, that exhibited the most variation. These spacers were then tested in additional isolates obtained from different geographic areas or different hosts or were implicated in different manifestations of human disease caused by *C. burnetii*. Thus, 173 *C. burnetii* isolates could be separated into 30 different genotypes. Phylogenetic analysis inferred from compiled sequences characterized three monophyletic groups, which could be subdivided into different clusters. In another study, Chmielewski et al. [32] used the MLST assay to characterize a limited number of selected *C. burnetii* isolates collected in Poland. The investigators used primer pairs that were described by Glazunova et al. [31]. Two sequence types were identified among six *C. burnetii* isolates examined, which might indicate only a slight heterogeneity of Polish isolates originating from different sources and regions.

The major advantage of the MLST assay lies in the fact that it allows an easy comparison and exchange of results obtained in different laboratories, and thus, it is a useful tool in identifying various *C. burnetii* isolates.

26.6 Infrequent Restriction Site: Silite PCR and Multiple Locus Variable Number Tandem Repeats Analysis

Recently, *C. burnetii* isolates of various origin have been typed using infrequent restriction site PCR (IRS-PCR) and multiple locus variable number tandem repeats (VNTR) analysis (MLVA). IRS-PCR has been shown to be a robust method for the molecular characterization of bacteria. The method starts with double digestion of genomic DNA using a combination of an infrequently and a frequently cutting RE. Following digestion, oligonucleotide adapters with specificity for the cleaved DNA ends are ligated. These adapters are subsequently used as primer binding sites for the PCR fragment amplification. Successful amplification produces a series of fragments that can be separated and visualized by GE. Once genome sequences are known, they can be interrogated for small repeat units that often vary between isolates.

In analyzing 14 *C. burnetii* isolates by four different IRS-PCR assays, the number of DNA fragments generated depended on the primers used and varied between six and ten [33]. The size of the amplicons varied between 100 and 1,000 bp. IRS-PCR assays using PsalG and PS1 primers generated the highest number of DNA fragments, whereas those using PsalC/PS1 or PsalT/PS1 generated the most diverse

patterns. The analysis was made in duplicate and little to no pattern variability between duplicate reactions was observed, only minor variations in the intensity of bands. It was found, however, that IRS-PCR may suffer on the inter-laboratory reproducibility problems inherent with multiple loci PCR amplifications.

Genotyping of bacteria through typing of loci containing VNTR might become the gold standard for many pathogens. The development of genome sequencing has shown that such sequences were present in every species analyzed and that polymorphism exists in at least a fraction of them. The length of these repetitions can vary from a single nucleotide to a few hundreds. This has implications for both the techniques used to measure the repeat number and the level of variability. In addition, tandem repeats can be part of coding regions or be intergenic and may play a direct role in the adaptation to the environment, thus having different observed evolution rates. For these reasons, the choice of VNTR when setting up the MLVA assay is important. Although reasonable discrimination can be achieved with the typing of six to eight markers, in particular in species with high genomic diversity, it may be necessary to type 20–40 markers in monomorphic species or if an evolutionary meaningful assay is needed.

Svraka et al. [34] developed the MLVA genotyping scheme using 16 isolates and five passage history/laboratory variants of *C. burnetii*. The whole genome sequence of the bacterium was screened for the presence of tandem repeats and a selection of eight different loci was made. The selection was based on the following criteria: the number of the repeats should be greater than four, the repeat size should not exceed 30 bp (this criterion was included so as to be able to analyze the sizes of the tandem repeats on agarose gels), and the conservation among the repeats should be more than 90%. Each VNTR locus was amplified using a forward primer labeled at the 5′ site with FAM and an unlabeled reverse primer. The separation of PCR fragments was performed using a DNA sequencer. Each isolate was assigned by an MLVA profile, defined by the number of repeats found at the different VNTR loci. Each unique MLVA profile was assigned an MLVA type. The VNTR markers revealed many polymorphisms resulting in nine unique MLVA types that cluster into five different clusters. The selected VNTR markers were stable. It has been suggested that the MLVA method is a promising tool for the characterization of *C. burnetii* isolates. In the subsequent study, Arricau-Bouvery et al. [33] identified 36 different genotypes among 42 *C. burnetii* isolates using MLVA. Two panels of markers were proposed: Panel 1, which can be confidently typed on agarose gel at lower cost and in any laboratory setting (ten minisatellite markers with a repeat unit larger than 9 bp), and Panel 2, which comprises seven microsatellites and provides a higher discriminatory power. The results of MLVA were in a good agreement with IRS-PCR.

In a number of instances, especially in species of recent origin, the discriminatory power of MLVA is much higher than that of MLST. The consistency of the results with independent methods indicates that MLVA can be applied for epidemiological studies. However, even if the underlying principles are relatively simple, quality standards must be implemented before this approach is widely accepted, and technology issues must be resolved to further lower the typing costs.

26.7 Conclusion

In this short review, an attempt has been made to present available typing methods for *C. burnetii* with a major focus on PCR-based techniques. Development of the PCR-based approaches for the analysis of amplified fragments allows convenient and rapid identification of the infectious pathogen.

Isolation of the bacterium provides a definitive diagnosis, but culturing of *C. burnetii* is a difficult, time-consuming task that requires a BSL 3 laboratory due to the organism's extreme infectivity and potential use as a weapon of bioterrorism. Serological methods are simpler and safer than the isolation methods. The most widely used serological test involves detection of anti- *C. burnetii* phase I and II antibodies. The test results can be confusing to those unfamiliar with Q fever. A combination of PCR and restriction analysis is much faster and by far a more sensitive assay for *C. burnetii* detection than the standard culture techniques. In addition, RT-PCR offers several advantages over the traditional detection techniques, including elimination of subjectivity and reduced labor, and the ability to standardize reporting of results is more repeatable and more sensitive.

MLST, IRS-PCR, and MLVA are methods of choice for systematic genotyping of *C. burnetii* isolates, each of them having its advantages and drawbacks. Nevertheless, these techniques will enhance considerably our ability to identify the source of infections and consequently help to reduce the number of cases in natural outbreaks or deliberate release events.

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